REGULATION OF SATELLITE CELL ACTIVITY AND IDENTITY

By

JU LI

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

1
ACKNOWLEDGMENTS

There are so many wonderful people who have helped me to complete this dissertation. First and foremost, I would like to thank my advisor, Dr. Sally Johnson, for her continuous support through my graduate studies. Over the past 6 years, her patience, genuine caring and concern, and faith in me encourage me to complete my research and dissertation. I also gratefully thank my talented and patient committee: Dr. Alan Ealy, Dr. Dwain Johnson, and Dr. David Criswell, for their invaluable academic support and input for my dissertation. I truly appreciate their assistance and commitment to my graduate study.

Special thanks also go to all my former and present lab mates: Xu Wang, Sarah Reed, Dane Winner, Jenelle McQuown, Sara and John Gonzalez, Shigeharu Tsuda, Wenli Sun, Walker Dillon, and Marni Lapin. Thank you all for sharing your skills, encouraging and friendship. I could not complete my dissertation without their help.

Last but most importantly, I thank my parents and my husband Bi, who provided unwavering love and support throughout all these years. They trust me, encourage me, and help me to become who I am today.
TABLE OF CONTENTS

page

ACKNOWLEDGMENTS .................................................................................................................. 3

LIST OF FIGURES ..................................................................................................................... 7

LIST OF ABBREVIATIONS ......................................................................................................... 10

ABSTRACT .................................................................................................................................. 13

CHAPTER

1 INTRODUCTION ......................................................................................................................... 15

Overview of Skeletal Muscle and Satellite Cells ................................................................. 15

Origin of Skeletal Muscle and Satellite Cells ................................................................. 16

Skeletal Myogenesis ............................................................................................................. 17

Pax7 and Pax3 ................................................................................................................................. 18

Myogenic Regulatory Factors (MRFs) .................................................................................. 20

Satellite Cell Markers ................................................................................................................ 22

Muscle-resident Stem Cell ...................................................................................................... 24

Satellite Cell ............................................................................................................................... 25

Satellite cell plasticity ............................................................................................................... 26

Satellite cell self-renewal .......................................................................................................... 27

Muscle Derived Stem Cell (MDSC) ...................................................................................... 29

Side-population (SP) cells ........................................................................................................ 29

Late-preplate (LP) cells .............................................................................................................. 30

Other Stem Cells ..................................................................................................................... 31

Satellite Cell Niche .................................................................................................................. 33

Extracellular Matrix (ECM) ................................................................................................... 34

Wnt ............................................................................................................................................... 35

Notch ........................................................................................................................................ 36

Myostatin .................................................................................................................................... 38

IGF-I ........................................................................................................................................... 38

FGF2 ........................................................................................................................................... 40

HGF ............................................................................................................................................ 41

Ephrin ........................................................................................................................................ 43

2 CENTRAL HYPOTHESIS .......................................................................................................... 46

3 HEPATOCYTE GROWTH FACTOR (HGF) SIGNALS THROUGH SHP2 TO REGULATE PRIMARY MOUSE MYOBLAST PROLIFERATION .......................................................... 47

Materials and Methods ........................................................................................................... 49

Satellite Cell Isolation and Cell Culture ................................................................................. 49

Plasmids and Cell Transfection .............................................................................................. 50
4 EVIDENCE OF HETEROGENEITY WITHIN BOVINE SATELLITE CELLS
ISOLATED FROM YOUNG AND ADULT ANIMALS ........................................ 78

Materials and Methods ........................................................................ 80
  Bovine Satellite Cell Isolation ............................................................ 80
  Cell Culture ..................................................................................... 80
  Immunocytochemistry ..................................................................... 81
  Statistics .......................................................................................... 82
Results ............................................................................................... 82
  BSCs Exhibit an Age-dependent Decline in Mitogenesis and Myogenesis. 82
  BSCs are a Heterogeneous Population .............................................. 82
  Extensive Degree of Heterogeneity Exists in Slow-growing Muscle Stem
  Cells .................................................................................................. 83
Discussion .......................................................................................... 85
  Animal Model for Satellite Cell Research ......................................... 85
  Heterogeneity of Bovine Satellite Cell .............................................. 86
  Asymmetric Division Independent of Satellite Cell Niche .................. 87
  Characteristics of Myf5-only Myoblast ............................................ 87
  Similarity of Bovine Satellite Cells and Human Satellite Cells ............ 89

5 EPHRIN-A5 REGULATES PRIMARY BOVINE SATELLITE CELLS
MIGRATION THROUGH RAC1/RHOA SIGNALING ................................. 108

Materials and Methods ........................................................................ 109
  Animal Care and Satellite Cells Isolation ......................................... 109
  Cell Culture ..................................................................................... 110
  Western Blot ................................................................................... 110
  Immunocytochemistry ................................................................... 111
  Time-lapse Photography .................................................................. 111
  Transwell Migration Assay .............................................................. 112
  Statistics .......................................................................................... 112
Results ............................................................................................... 112
  Bovine Satellite Cells Exhibit Variant Pattern of Motility in vitro ....... 112
  Ephrin-A5 is an Attractive Cue for BSCs Migration ............................ 113
  Only Muscle Progenitors are Affected Prior to Activation ................. 114
  Ephrin-A5 Regulates BSCs Migration Independent of ERK1/2 Signaling. 115
  Ephrin-A5 Regulates BSCs Migration through Rac1/RhoA Signaling. .... 116
Discussion .......................................................................................... 117

6 IMPLICATIONS .............................................................................. 144
### APPENDIX

A  SATELLITE CELL ISOLATION PROTOCOL .......................................................... 147
B  SDS-PAGE AND WESTERN BLOT PROTOCOL .................................................. 149
C  IMMUNOCYTOCHEMISTRY PROTOCOL ............................................................ 151
D  TIME-LAPSE PHOTOGRAPHY PROTOCOL ....................................................... 152
E  TRANSWELL MIGRATION ASSAY PROTOCOL .................................................. 153

LIST OF REFERENCES ......................................................................................... 154

BIOGRAPHICAL SKETCH ...................................................................................... 181
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>All SHP2 myoblasts cell lines express abundant amounts of fusion proteins</td>
<td>63</td>
</tr>
<tr>
<td>3-2</td>
<td>SHP2 stimulates proliferation in myoblasts</td>
<td>64</td>
</tr>
<tr>
<td>3-3</td>
<td>Activated SHP2 inhibits myofiber formation</td>
<td>65</td>
</tr>
<tr>
<td>3-4</td>
<td>Activated SHP2 inhibits myogenin expression</td>
<td>66</td>
</tr>
<tr>
<td>3-5</td>
<td>Hepatocyte growth factor (HGF) induced proliferation requires SHP2</td>
<td>67</td>
</tr>
<tr>
<td>3-6</td>
<td>Hepatocyte growth factor (HGF) stimulates ERK1/2 activation independent of</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>SHP2</td>
<td></td>
</tr>
<tr>
<td>3-7</td>
<td>Hepatocyte growth factor (HGF) and SHP2 stimulate proliferation</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>independent of ERK1/2</td>
<td></td>
</tr>
<tr>
<td>3-8</td>
<td>SHP2 does not affect satellite cell proliferation and activation</td>
<td>70</td>
</tr>
<tr>
<td>3-9</td>
<td>Mouse satellite cells (MSCs) expresses a higher concentration of phospho-</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>SHP2 than 23A2 myoblasts</td>
<td></td>
</tr>
<tr>
<td>3-10</td>
<td>SHP2 protein expression was knockdown by SHP2 siRNA</td>
<td>72</td>
</tr>
<tr>
<td>3-11</td>
<td>Blockage of SHP2 promotes satellite cell proliferation</td>
<td>73</td>
</tr>
<tr>
<td>3-12</td>
<td>High concentrations of HGF induced arrest of mouse satellite cells</td>
<td>74</td>
</tr>
<tr>
<td>3-13</td>
<td>HGF induced arrest is mediated by SHP2</td>
<td>75</td>
</tr>
<tr>
<td>3-14</td>
<td>HGF induces ERK1/2 activation independent of phosphor-SHP2</td>
<td>76</td>
</tr>
<tr>
<td>3-15</td>
<td>HGF induced arrest requires ERK1/2 activation</td>
<td>77</td>
</tr>
<tr>
<td>4-1</td>
<td>Bovine satellite cells exhibit an age-dependent reduction in cell growth</td>
<td>91</td>
</tr>
<tr>
<td>4-2</td>
<td>Bovine satellite cells exhibit an age-dependent decline in proliferation</td>
<td>92</td>
</tr>
<tr>
<td>4-3</td>
<td>Bovine satellite cells display an age-dependent decline in MyoD expression</td>
<td>93</td>
</tr>
<tr>
<td>4-4</td>
<td>Bovine satellite cells display an age-dependent decline in myogenin</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>expression</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>Bovine satellite cells are composed of three sub-populations based on Pax7</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>and Myf5 expression</td>
<td></td>
</tr>
</tbody>
</table>
Bovine satellite cells from young and adult animals have similar percentage of Pax7-only cells ................................................................. 96
Bovine satellite cells from young animals exhibit a faster decline of Pax7:Myf5 dual-positive cell percentage ................................................. 97
Bovine satellite cells from young animals exhibit a higher percentage Myf5-only cell ................................................................. 98
BSCs heterogeneity varies in different muscles ........................................ 99
Bovine satellite cells have different growth rates .................................. 100
Bovine satellite cells contain two sub-types based on different growth rates ... 101
Extensive degree of heterogeneity exists among slow-growing colonies ........ 102
Pax7 expression muscle stem cell divided through both symmetric and asymmetric manner ................................................................. 103
Pax7 expression muscle stem cell divided through both symmetric and asymmetric manner ................................................................. 104
Myf5-only cells proliferate faster than Pax7-only cells ......................... 105
Myf5-only cells do not differentiate or fuse to myofibers ...................... 106
Bovine satellite cells are heterogeneous populations .......................... 107
Bovine satellite cells have variable motility ........................................ 122
Bovine satellite cells have variable motility ........................................ 123
Slow-moving BSCs are living cells that capable of stretching .............. 124
EphA2 and EphA4 are expressed in 23A2 myoblasts and bovine satellite cells (BSCs) ................................................................. 125
Ephrin-A5 binds to its receptor on the cell membrane .......................... 126
Ephrin-A5 changes motility of bovine satellite cells (BSCs) .................... 127
Multimeric Ephrin-A5 acts as a chemo-attractant for bovine satellite cells (BSCs) ................................................................. 128
Dimeric Ephrin-A5 acts as a chemo-attractant for BSCs ....................... 129
Ephrin-A5 only affects muscle progenitors ........................................ 130
5-10 Ephrin-A5 only affects BSCs prior to long-term culture ........................................ 131
5-11 Bovine satellite cells express MyoD after long-term of culture .......................... 132
5-12 Ephrin-A5 activates ERK1/2 in 23A2 myoblasts .................................................. 133
5-13 Ephrin-A5 does not activate ERK1/2 or Akt in bovine satellite cells (BSCs) .... 134
5-14 PD98059 causes blockage of ERK1/2 activation ............................................... 135
5-15 Blockage of ERK1/2 activation does not affect ephrin-A5-induced migration of bovine satellite cells (BSCs) .......................................................... 136
5-16 HGF activates ERK1/2 activation ........................................................................ 137
5-17 HGF changes motility of BSCs after 24 hours of treatment .............................. 138
5-18 HGF promotes migration of bovine satellite cells (BSCs) ................................. 139
5-19 Ephrin-A5 promotes BSCs migration through Rac1/RhoA signaling .............. 140
5-20 Ephrin-A5 is internalized in bovine satellite cells (BSCs) and 23A2 myoblasts ................................................................................................................. 141
5-21 Ephrin-A5 internalization is delayed in bovine satellite cells (BSCs) compared to 23A2 myoblasts ................................................................................. 142
5-22 Rac1/RhoA signaling does not affect Ephrin-A5 internalization in bovine satellite cells (BSCs) .................................................................................. 143
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker muscular dystrophy</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSC</td>
<td>bovine satellite cell</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>E₂</td>
<td>estradiol-17β</td>
</tr>
<tr>
<td>ECL</td>
<td>entactin-collagen-laminin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Gab1</td>
<td>Grb-associated binder 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GDF</td>
<td>growth/differentiation factor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LD</td>
<td>longissimus dorsi</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LRP</td>
<td>lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDSC</td>
<td>muscle-derived stem cell</td>
</tr>
<tr>
<td>MMP</td>
<td>metalloproteinase</td>
</tr>
<tr>
<td>MNF</td>
<td>myocyte nuclear factor</td>
</tr>
<tr>
<td>MRF</td>
<td>myogenic regulatory factor</td>
</tr>
<tr>
<td>MyHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>N-CAM</td>
<td>neural cell adhesion molecular</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDT</td>
<td>population doubling time</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferators-activated receptor</td>
</tr>
<tr>
<td>PTPN11</td>
<td>protein tyrosine phosphatase non-receptor type 11</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SF</td>
<td>scatter factor</td>
</tr>
<tr>
<td>SHP2</td>
<td>Src homology 2-containing protein tyrosine phosphatase 2</td>
</tr>
<tr>
<td>SHPS-1</td>
<td>SHP substrate-1</td>
</tr>
<tr>
<td>SM</td>
<td>semimembranosus</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TBA</td>
<td>trenbolone acetate</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor- β</td>
</tr>
<tr>
<td>V-CAM</td>
<td>vascular cell adhesion molecular</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Satellite cells are the tissue-specific stem cells responsible for skeletal muscle growth and repair. During postnatal muscle growth and muscle injury, satellite cells activate, proliferate, migrate, differentiate, and fuse to muscle fibers. Many growth factors and chemokines are involved in regulation of satellite cell proliferation and migration. The objective of this research is to characterize satellite cells and committed myoblasts from different species and identify factors that regulate satellite cell activity.

Niche localized hepatocyte growth factor (HGF) plays an integral role in G0 exit and return to mitotic activity of adult satellite cells. HGF signaling regulates satellite cell activity through recruitment of SHP2, a protein tyrosine phosphatase. Both HGF treatment and SHP2 overexpression increased proliferation of 23A2 myoblasts. Importantly, the effects of SHP2 are independent of downstream ERK1/2 activity. Functions of HGF and SHP2 were further evaluated in primary mouse satellite cells. Low concentrations of HGF promote proliferation, whereas high concentrations of HGF inhibit proliferation, which can be overcome by knockdown of SHP2 with siRNA. These results suggest that HGF signals through SHP2 in myoblasts and mouse satellite cells to directly alter proliferation rates.
To better understand the satellite cell activity in meat-producing animals, bovine satellite cells (BSCs) from skeletal muscle of young and adult cattle were isolated and analyzed. Similar to rodents, BSCs exhibit an age-dependent delay in proliferation and differentiation. Immunocytochemistry and clonal analysis reveals that BSCs are a heterogeneous population composed of at least three distinct subgroups. Pax7-only cells and Pax7/Myf5 double-positive cells correspond to mouse muscle stem cells and committed progenitors, respectively. Interestingly, a unique subgroup of Myf5-only cells was identified in BSCs that may represent a Pax3-expressing subpopulation.

Further analysis of BSC motion demonstrates that different subpopulations of cells exhibit distinct motilities. During muscle development, ephrin-A5 is required for migration of myogenic precursors to the limb area. However, function of ephrin-A5 in the migration of postnatal satellite cells is still unclear. Ephrin-A5 appears to regulate motility of BSCs in a chemo-attractive manner. However, only a subgroup of muscle progenitor cells responds to ephrin-A5-induced migration. Furthermore, the ephrin-A5 action on cell migration is directly through downstream Rac1/RhoA signaling. These results suggest ephrin-A5 acts as a chemoattractant for postnatal BSCs, and define the intracellular signal involved in ephrin-A5-induced migration of BSCs.

In summary, this study clarifies the bovine satellite cell populations, and the modulation of satellite cell activity through extrinsic regulation factors. As a result, study of satellite cell identity and activity from the meat-producing animals is the primary step to understand the molecular mechanism of postnatal muscle growth and hypertrophy.
Overview of Skeletal Muscle and Satellite Cells

Skeletal muscle is the most abundant tissue of the human body and accounts for 30-40% of total body mass (Sandow, 1970). It is also one of the most stable tissues in the body, with a very low protein turnover rate (Millward et al., 1975; Mittendorfer et al., 2005; Spalding et al., 2005). However, when stimulate by exercise or muscle injury, skeletal muscle is able to regenerate itself rapidly.

Skeletal muscle regeneration depends mainly on satellite cells (SCs), a type of adult muscle stem cells. The satellite cell is named after its localization, between plasma membrane of the muscle cell and basal lamina that surrounds each muscle fiber (Mauro, 1961). These cells exist in multiple species, including rodent and human (Cardasis and Cooper, 1975; Schmalbruch and Hellhammer, 1976). However, their number and characteristic vary with animal species, age and muscle types. Mouse satellite cells represent about 30% of all nuclei in hindlimb muscle at birth. After weaning, the percentage rapidly drops to 4% in adult mice and 1-2% in aged mice (Cardasis and Cooper, 1975; Snow, 1977). The age-associated decline in the percentage of satellite cells partially explains the decreased efficiency of muscle regeneration in older subjects. Satellite cells from aged animals also display reduced proliferation and differentiation activities (Charifi et al., 2003).

Although satellite cells stay in a quiescent state and represent very low percentage of all nuclei in adult muscle, upon injury, they are able to activate and give rise to large numbers of myogenic cells within a few days (Tedesco et al., 2010). Thus, satellite cells are considered to be the major player in postnatal growth and regeneration of skeletal
muscle. They contribute to terminal differentiated myofibers, as well as replenish stem cell pool through self-renewal (Zammit et al., 2004).

**Origin of Skeletal Muscle and Satellite Cells**

In vertebrates, all skeletal muscles are derived from paraxial mesoderm during embryonic development (Shih et al., 2008). However, different muscles have distinct origins. Dermomyotome, the dorsal part of somite (an epithelial structures derived from the paraxial mesoderm), forms muscles of the trunk and limbs. The medial dermomyotome gives rise to the skeletal muscles in the back of the body (epaxial musculature), whereas lateral dermomyotome gives rise to trunk and limb muscles (hypaxial musculature) (Ordahl and Le Douarin, 1992; Tajbakhsh and Cossu, 1997).

The majority of the head muscles are from anterior, unsegmented, paraxial mesoderm and prechordal mesoderm (Buckingham et al., 2003).

The majority of satellite cells are derived from the somite. However, there are different opinions concerning the developmental origin of satellite cells (Messina and Cossu, 2009; Ono et al., 2010). In one theory, satellite cells are considered as the remnant embryonic myogenic progenitors derived from dermomyotome. Embryonic myogenic progenitors delaminate from the dermomyotome. Majority of the progenitors migrate into the limb buds to form limb muscle, while minority lay aside of muscle for future use (Messina and Cossu, 2009). These embryonic progenitors are derived from multipotent mesodermal cells, which express transcription factors Pax3 and Pax7 (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). The Pax3/Pax7-positive cells are committed to the myogenic lineage in response to signals from axial tissues, such as different Wnt signals (Wnt 1/4/5a/6/7a) from neural tube and Sonic Hedgehog from the notochord (Munsterberg and Lassar,
These cells start to express the myogenic determination factors, Myf5 and MyoD, to commit to myogenic lineage (Bober et al., 1994; Goulding et al., 1994). These committed cells migrate to the subjacent myotome, express the differentiation factors myogenin and MRF4, and fuse to form myofibers (Pownall et al., 2002). MRF4 is transiently expressed at an earlier embryonic stage followed by up-regulation during late fetal development. The expression pattern suggests MRF4 may have additional functions in early embryonic muscle maturation (Zhang et al., 1995).

**Skeletal Myogenesis**

Satellite cells are identified as myogenic stem cells that contribute to muscle growth, repair, and regeneration (Bischoff, 1975; Konigsberg et al., 1975). They reside in adult healthy skeletal muscle at a quiescent state. In response to muscle stretch, hormone stimulation, or muscle injury, the quiescent satellite cells start to activate and go through myogenesis (Morgan and Partridge, 2003). These activated satellite cells are stimulated, re-enter the cell cycle, and give rise to a pool of muscle precursor cells, which undergo multiple rounds of proliferation prior to terminal differentiation and fusion with new or growing myofibers (Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994). During muscle injury, several signals derived from both damaged fibers and infiltrating cells are involved in SC activation, including hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and nitric oxide (NO).

After satellite cell activation and proliferation, a small amount of cells do not undergo terminal differentiation. Instead, they withdraw from cell cycle and go back to quiescent state to restore the reserve pool of muscle stem cells (Zammit et al., 2004).
Satellite cells are considered adult muscle stem cells due to the self-renewal characteristic and myogenesis capability (Morgan and Partridge, 2003).

**Pax7 and Pax3**

Pax7 and Pax3 are members of the paired box family of transcription factors. They have some common functions for progenitor cell survival and specification during embryonic development. Almost all dermomyotomal cells express Pax3 and Pax7 at early embryonic stage, and then Pax3 is preferentially expressed in the dorsal and ventral dermomyotome lips, whereas Pax7 is highly expressed in the central dermomyotome (Kassar-Duchossoy et al., 2005). Pax3 and Pax7 expression defines a population of myogenic progenitors, which give rise to satellite cells at late stage. In the absence of both Pax3 and Pax7, these progenitors go through apoptosis or alternative non-myogenic cell fates (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). However, knockout of Pax3 or Pax7 in mice induces different phenotypes, which suggests that Pax3 and Pax7 have some distinct functions and are not able to replace each other (Kuang et al., 2006; Relaix et al., 2006; Relaix et al., 2004).

Pax7 is the most widely used marker for satellite cells, which is expressed in both quiescent and activated satellite cells across different species, such as human, monkey, mouse, pig, chick, salamander and zebrafish (Kuang and Rudnicki, 2008). Pax7 is required for satellite cell specification and survival during embryonic development. Prenatal ablation of Pax7 in mice causes a defect in cephalic neural crest, as well as muscle atrophy. These mice are completely absent of satellite cells, exhibit 50% reduction in body weight at 7 days, and usually die within 2 weeks after birth (Mansouri et al., 1996; Seale et al., 2000). The CD45⁺/Sca1⁻ stem cells exist in Pax7-null muscle; however, they do not undergo myogenesis unless exposed to Wnt signaling. When
Pax7 expression is induced in these CD45+/Sca1+ cells, their myogenic potential is restored (Polesskaya et al., 2003; Seale et al., 2004). Some Pax7 knockout mice are able to survive to juvenile or adulthood. They contain reduced but sustained number of satellite cells, which maintain the number and size of skeletal muscle. However, the muscle regeneration ability is impaired in these Pax7 mutant mice (Oustanina et al., 2004). Recently, Lepper et al. (2009) used an inducible Cre/loxP system to examine the function of Pax7 during myogenesis in embryonic, juvenile and adult skeletal muscles. Contrary to the embryonic Pax7 knockout mice, Pax7 inactivation in adult mice does not affect satellite cell regeneration. The satellite cells can proliferate and reoccupy the sublaminal satellite cell niche, as well as support further regeneration process (Lepper et al., 2009). Furthermore, multiple time points of Pax7 inactivation reveals that Pax7 is only required before juvenile period, when myogenic progenitor cells make the transition into quiescence (Lepper et al., 2009). These results demonstrate that Pax7 plays an essential role in myogenic specification during embryonic development, but is not necessary for adult satellite cell activity.

Pax3 shares substantial sequence homology with Pax7 and is required for embryonic neurogenesis and myogenesis (Goulding et al., 1991; Schafer et al., 1994). Pax3 is transcribed in paraxial mesoderm prior to segmentation, and required for the delamination and migration of myogenic progenitor cells (Buckingham and Relaix, 2007). Quiescent satellite cells from many, but not all muscle groups express Pax3 (Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Relaix et al., 2006). For example, Pax3 is expressed in diaphragm, most trunk muscles, and some limb muscles. But in lower hindlimb muscles, such as tibialis anterior (TA) muscle, Pax3 expression is
dramatically reduced during the late fetal stages (Relaix et al., 2006). The uneven expression of Pax3 suggests that satellite cells in different muscles may derive from different parts of the somite.

Splotch (Sp) mutant mice lacking functional Pax3 display impaired migration of somitic muscle precursors into the developing limb buds (Bober et al., 1994). As a result, Pax3-null mice fail to develop the limb muscle, but make normal trunk muscle (Bober et al., 1994; Goulding et al., 1994). In addition, mice with double mutation of Sp and Myf-5 do not express MyoD in their somites, suggesting that Myf-5 and Pax3 work upstream of MyoD in myogenic determination (Tajbakhsh et al., 1997). Ectopic expression of Pax3 in mesodermal explants of avian embryonic tissue induces the expression of MyoD and Myf-5, which suggests Pax3 is sufficient to activate Myf-5 and MyoD, thereby initiates skeletal myogenesis (Maroto et al., 1997). Furthermore, similar to the adult Pax7 inactive mice, adult Pax3/Pax7 dual-inactive mice also exhibit normal skeletal muscle with unaffected regeneration capability, which indicates neither Pax3 nor Pax7 is necessary for adult myogenesis (Lepper et al., 2009).

**Myogenic Regulatory Factors (MRFs)**

The MRFs (Myf5, MyoD, myogenin, and MRF4) are a group of transcription factors that control the specification and differentiation of myogenic lineage, during both prenatal and postnatal muscle development. MRF proteins share a conserved DNA binding domain and a basic helix-loop-helix (bHLH) motif for heterodimerization with E proteins, a second bHLH protein family. E-protein family, including E12, E47, and HEB, are important to regulate MRF activity during myogenesis (Ludolph and Konieczny, 1995). MRF-E protein dimmer binds DNA at E-box (CANNTG), which is located in the
promoters of many skeletal muscle specific genes and mediates gene activation in an MRF-dependent manner (Sabourin and Rudnicki, 2000).

MRFs mRNAs are expressed in a temporally distinct pattern during mouse embryonic development. For example, Myf5 is first activated around 8 days postcoitum (dpc) in epaxial somitic cells. MyoD is activated later at 10.5 dpc, when Myf5 expression starts to drop (Ott et al., 1991; Sassoon et al., 1989). Myf5 and MyoD are also expressed in the muscle progenitor cells that migrate from dermomyotome to hypaxial and limb muscle (Sassoon et al., 1989). Their expression is mediated through Pax3 activation (Maroto et al., 1997; Tajbakhsh et al., 1997). The activation of myogenin is observed at 8.5 dpc along with markers of terminal differentiation (Sassoon et al., 1989). MRF4 is expressed transiently between day 9 and 12, and then repressed until after birth (Bober et al., 1991).

MRFs play a significant role in myogenic lineage determination and differentiation. MyoD and Myf5 are first identified with their ability to transform other cell types to myogenic lineage (Braun et al., 1989; Davis et al., 1987). Inactivation of MyoD in mice results in no change in phenotype other than a four-fold increase in Myf-5 expression (Rudnicki et al., 1992). Similarly, Myf-5 deficient mice display normal skeletal muscle but die within minutes of birth because of severe rib defects, with no change of MyoD expression (Braun et al., 1992). Mice deficient of both MyoD and Myf-5 die at birth due to complete absence of myoblasts (Rudnicki et al., 1993). Furthermore, muscle development in hypaxial musculature (limb and abdominal wall muscle) is delayed for 2.5 days in MyoD knockout mice; and Myf5-null mice display delayed development of epaxial musculature (paraspinal and intercostals muscle). The different resulted
phenotypes suggest Myf5 and MyoD have distinct function in regulating limb and trunk muscles (Kablar et al., 1997). MRF4 and myogenin are identified through their function in regulating terminal myogenic differentiation (Braun et al., 1990; Edmondson and Olson, 1989; Miner and Wold, 1990; Rhodes and Konieczny, 1989; Wright et al., 1989). Myogenin knockout mice display normal number of myoblasts but die at birth because of absence of myofibers (Hasty et al., 1993; Nabeshima et al., 1993). By contrast, inactivation of MRF4 results in viable mice with apparently normal skeletal muscle but an increase in myogenin expression (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995).

Taken together, these results indicate that myogenin and MRF4 are required for differentiation of myoblasts into myofibers, and myogenin can substitute for MRF4 without affecting muscle development. MRF4 is transient transcribed during early embryonic development prior to MyoD and myogenin expression (Zhang et al., 1995). In addition, expression of MRF4 in adult animals leads to expression of MyoD and myogenin (Rhodes and Konieczny, 1989). These results demonstrates that MRF4 acts upstream of MyoD and myogenin.

**Satellite Cell Markers**

Satellite cells are initially defined by their location. Further studies suggest that the expression of some specific proteins can be used as markers for satellite cell as well.

Major protein markers identified in satellite cells contain four categories:

1. Transcription factors, including Pax3 (Montarras et al., 2005; Relaix et al., 2006), Pax7 (Seale et al., 2000), Myf5 (Beauchamp et al., 2000; Tajbakhsh and Buckingham, 1995).

2. Intermediate filaments, including Nestin (Day et al., 2007), Desmin (Asakura et al., 2001).
3. Transmembrane receptors, including c-met (Cornelison and Wold, 1997; Wozniak et al., 2003), integrin α7 (Blanco-Bose et al., 2001; Gnocchi et al., 2009), integrin β1 (Cerletti et al., 2008; Sherwood et al., 2004), CXCR4 (Cerletti et al., 2008; Sherwood et al., 2004).


Some of these markers can be used to trace satellite cells or purify subpopulations of satellite cells during embryonic development or adult myogenesis. For example, Pax3-GFP reporter shows Pax3-positive embryonic muscle progenitor cells adopt satellite cell position at late fetal stage (Relaix et al., 2005). Combination of Myf5-LacZ and Myf5-Cre/ROSA-YFP reporters is also used to identify stem cell compartment within satellite cells that never express Myf5 (Kuang et al., 2007). Furthermore, since most satellite cell markers are not exclusively expressed in satellite cells, a combination of different markers are used to purify satellite cells by fluorescence-activated cell sorting (FACS) (Cerletti et al., 2008; Fukada et al., 2004; Montarras et al., 2005; Pawlikowski et al., 2009).

Research of satellite cell markers provides a good view of both the embryonic development and the postnatal progression of skeletal muscle and satellite cells. For example, CD34 is only expressed in quiescent satellite cells, and Myf5 and M-cadherin are only expressed in committed precursors. Early feature of satellite cell activation is alternate splicing followed by complete transcriptional shutdown of CD34 (Beauchamp et al., 2000). In addition, different species have some distinct expression patterns of satellite cell markers. CD34, M-cadherin and desmin are three of the most reliable markers for satellite cells in mice (Asakura et al., 2001; Beauchamp et al., 2000;
Cornelison and Wold, 1997; Irintchev et al., 1994); however, there is no evidence showing CD34 expression in human, while M-cadherin and desmin are not expressed in every human satellite cell (Pawlikowski et al., 2009; Sajko et al., 2004). The most reliable marker of satellite cells in human muscle is CD56, also called N-CAM (Lindstrom and Thornell, 2009; Schubert et al., 1989). A recent study found that c-met and α7-integrin could be used as purification markers of human satellite cells. In addition, desmin and MyoD expression is an indicator of human satellite cell lineage progression (Pawlikowski et al., 2009).

**Muscle-resident Stem Cell**

Research over the past 10 years has shown the presence of several stem cell populations in skeletal muscle. Generally, stem cells are characterized by three properties: maintaining long-term proliferation during unspecialized stage, self-renewal, and differentiation into multiple mature cell types (plasticity). In skeletal muscle, there are multiple types of stem cells: satellite cells (committed stem cells which function as myogenic precursors), muscle-derived stem cells (MDSCs, populations of multipotent adult stem cells), and some other types of stem cells that can enter myogenic lineage. Different types of muscle-resident stem cells are described later. Satellite cells and MDSCs are distinct cell populations. Based on their location, satellite cells locate within the basal membrane of muscle fiber, but MDSCs stay outside the muscle fiber. Satellite cells and MDSCs also have different marker expression. For example, MDSCs are positive for hematopoietic stem cell marker Sca-1 but negative for satellite cell markers Pax7. In opposite, satellite cells are negative for Sca-1 and positive for Pax7 (Asakura et al., 2002). Moreover, satellite cells automatically go through myogenesis, and there is no evidence for satellite cells transdifferentiation to other cell types in vivo. MDSCs are
capable of differentiating into regenerated muscle fiber during intra-arterial or intramuscular transplantation. However, the default differentiation pathway of MDSCs is not myogenic, since they preferentially differentiate to hematopoietic cell in vivo. Thus, MDSCs are multipotent stem cells, whose myogenic potential is probably regulated via a SC-dependent mechanism (Asakura et al., 2002; Gussoni et al., 1999).

**Satellite Cell**

Satellite cells are a heterogeneous population composed of both committed myogenic progenitor cells and stem cells. Beauchamp et al (2000) found CD34, Myf5 and M-cadherin co-localized in 80% of satellite cells. The same group further studied the relationship between marker expression and cell function, which demonstrated majority of cells co-expressed of Myf5 and M-cadherin. The cells were defined to be quiescent, committed myogenic precursor cells. The double negative cell did not express MyoD and failed to fuse into muscle fibers, which suggested that they might be stem cells involved in maintaining the myogenic precursors (Zammit et al., 2002). Further studies were carried out to characterize or purify the stem cell population within mouse or human satellite cells through different methods and techniques, such as FACS by cell surface marker (Cerletti et al., 2008; Fukada et al., 2004; Pawlikowski et al., 2009), lineage tracing (Kuang et al., 2007; Montarras et al., 2005), as well as cell shape and growth rate analysis (Hashimoto et al., 2004; Rouger et al., 2004). The stem cells obtained from different methods share some common characteristics, but they may not be exactly the same population (Seale and Rudnicki, 2000). Moreover, satellite cell heterogeneity also varies with animal age, muscle groups and muscle condition (Collins et al., 2007; Lindstrom and Thornell, 2009; Seale et al., 2004).
Lineage tracing of mouse satellite cells (MSCs) shows that 90% of MSCs are committed myogenic progenitor cells with both Pax7 and Myf5 expression, and 10% of MSCs are myogenic stem cells that never express Myf5. Transplantation of these cells into muscle reveals that Pax7+/Myf5+ progenitors exhibit myogenic differentiation, and Pax7+/Myf5− stem cells contribute to keep satellite cell reservoir (Kuang et al., 2007). In human studies, three sub-types of cells are identified in satellite cells using NCAM and Pax7 staining: NCAM+/Pax7+ (94%), NCAM+/Pax7− (5%), and NCAM−/Pax7+ (1%). While majority of satellite cells that expressing both markers are muscle progenitors; NCAM+/Pax7− and NCAM−/Pax7+ cells represent the myoblast and stem cell populations separately (Lindstrom and Thornell, 2009). Thus, mouse satellite cells and human satellite cells have different marker expression pattern, which suggest they contain distinct sub-population of cells.

**Satellite cell plasticity**

While satellite cells are traditionally thought to be committed myogenic precursors, there is evidence suggesting that satellite cells are multipotent with alternative differentiation potentials in vitro (Asakura et al., 2001; Lee et al., 2000; Teboul et al., 1995b; Wada et al., 2002). For example, bone morphogenetic protein 2 (BMP2) treatment activates the osteogenic pathway of C2C12 myoblasts, an immortalized cell line derived from mouse limb muscle (Fux et al., 2004b; Katagiri et al., 1994; Yamaguchi et al., 1991). Treatment with thiazolidinediones and fatty acids converts C2C12 cells to the adipogenic cell fate (Teboul et al., 1995a). Additionally, primary mouse satellite cells from adult muscle perform similarly as myoblasts in the presence of strong adipogenic inducers, such as CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferators-activated receptor γ (PPARγ) (Asakura et al., 2001; Fux et
al., 2004b; Hu et al., 1995). Interestingly, mouse satellite cells isolated from myofiber
give rise to both myogenic and non-myogenic clones spontaneously (Shefer et al.,
2004). It is found that undifferentiated cells in adult myoblast cultures co-express MyoD,
Runx2, and PPARγ, which are key regulators of myogenesis, osteogenesis, and
adipogenesis respectively. The result supports the hypothesis that satellite cells have a
multipotential predisposition (Fux et al., 2004a; Wada et al., 2002). Furthermore, muscle
degeneration (Akimoto et al., 2005; Hosoyama et al., 2009), denervation (Dulor et al.,
1998) or aging (Ross et al., 2000; Taylor-Jones et al., 2002) can induce satellite cells to
adopt divergent lineages. However, there is no direct evidence for satellite cell
transdifferentiation in normal conditions in vivo. In brief, the non-myogenic potency of
satellite cells is intrinsically limited in the healthy muscle by some regulators, such as
Wnt 10b (Taylor-Jones et al., 2002; Vertino et al., 2005). By contrast, MDSCs can
establish a muscle, neural, bone or endothelial lineage both in vitro and in vivo (Lee et
al., 2000; Qu et al., 1998; Qu-Petersen et al., 2002).

**Satellite cell self-renewal**

When a stem cell divides, one daughter cell is a committed specialized cell, while
the other remains an unspecialized stem cell (Dhawan and Rando, 2005). As muscle
regenerates, quiescent satellite cells become activated and enter G₁ of the cell cycle
(Charge and Rudnicki, 2004; Morgan and Partridge, 2003). These highly proliferative
progenitor cells down-regulate Pax7, up-regulate MyoD, and ultimately form
differentiated myofibers (Charge and Rudnicki, 2004; Schultz, 1996; Schultz and
McCormick, 1994). However, the number of satellite cells remains constant through
muscle regeneration, which suggests satellite cells replenish the quiescent cell pool
through cell-renewal (Dhawan and Rando, 2005; Schultz and McCormick, 1994).
During cell division of myoblasts or satellite cells, two daughter cells have distinct expression of myogenic lineage markers. One daughter cell expresses activated satellite cell markers, such as MyoD and Myf5, and the other cell expresses quiescent cell markers, such as Pax7 and desmin, which indicates that they adopt divergent cell fate (Baroffio et al., 1996; Olguin and Olwin, 2004; Yoshida et al., 1998). Using lineage markers to trace a single quiescent cell associated with myofiber demonstrates the asymmetric division and self-renewal of satellite cells (Zammit et al., 2004). Quiescent satellite cells in the myofiber co-express the transcription factor Pax7 and MyoD during activation. Most of the proliferating cells down-regulate Pax7 and differentiate; whereas minority of proliferating cells maintain Pax7 expression, lose MyoD expression, and further withdraw from immediate differentiation. Moreover, the results of tracing single myofiber transplanted in vivo show single myofiber can generate over 100 new myofibers; and the donor satellite cells give rise to new myonuclei and repopulate the host satellite cell pool (Collins et al., 2005). Further analysis of the muscle stem cells through Myf5 lineage tracing reveals that these Pax7+/Myf5- cells give rise to committed progenitors through apical-basal oriented asymmetric division to generate a basal Pax7+/Myf5- stem cell and an apical Pax7+/Myf5+ progenitor (Kuang et al., 2007).

The molecular mechanism of satellite cell asymmetric division and self-renewal remains unclear. A fraction of satellite cells are capable of segregating the older parental chromosomes with the self-renewing cell away from the newly replicated DNA of the daughter cell (Conboy et al., 2007; Shinin et al., 2006). Numb, a Notch signaling inhibitor, also asymmetrically segregates with the daughter cells, suggesting Notch is important for self-renewal and fate determination (Conboy and Rando, 2002). In
addition, activation of Notch-1 promotes the expression of quiescent marker Pax3 in proliferating satellite cells, whereas Numb expression leads to commitment of progenitor cells to myoblast cell fate (Conboy and Rando, 2002). Beta-catenin, the Wnt signal effector, promotes satellite cells to express of Pax7 without MyoD, therefore make satellite cells to adopt the self-renewal fate (Perez-Ruiz et al., 2008). Thus, both Notch and Wnt signalings regulate the satellite cell self-renewal and fate determination.

**Muscle Derived Stem Cell (MDSC)**

Similar to satellite cells, MDSCs reside in skeletal muscle and contribute to both myofiber differentiation and myogenic cell repopulation. Different from satellite cells, MDSCs locate outside the basal membrane, is absent of Pax7 expression, and maintain multipotency (Peng and Huard, 2004). Based on different isolation and purification methods, MDSCs can be separated to two groups: side population (SP) cells (isolated based on Hoechst dye efflux) and late preplate (LP) cells (isolated based on slow adhesion).

**Side-population (SP) cells**

Side-population (SP) cells are a population of pluripotent stem cells isolated based on Hoechst dye efflux, which is first identified in murine bone marrow (Ferrari et al., 1998; Goodell et al., 1996; LaBarge and Blau, 2002). Fluorescence-activated cell sorting (FACS) analysis demonstrates that there is a fraction of SP cells associated with the skeletal muscle fibers (Oustanina et al., 2004; Seale et al., 2000; Seale et al., 2004). Skeletal muscle SP cells express the stem cell marker Sca-1 and c-kit, but not the myogenic marker Pax7 (Asakura et al., 2002; Gussoni et al., 1999; Jackson et al., 1999). These SP cells have the capacity to contribute to skeletal muscle regeneration following intravenous injection into irradiated mice (Gussoni et al., 1999). Further
experiments show these cells are able to go through both myogenic and hematopoietic
differentiation pathways, and give rise to myofibers as well as all major blood lineages
(Jackson et al., 1999).

When introduced into lethally irradiated mice, muscle SP cells are able to
regenerate skeletal muscle and give rise to satellite cells (Asakura et al., 2002; Gussoni
et al., 1999; Jackson et al., 1999). However, experiment shows the muscle SP cells are
located outside of the basal membrane of myofibers, absent of myogenic marker
expression (such as Pax7, Myf5), and exhibit limited myogenic capability in vitro (Seale
et al., 2000). For example, using Myf5-lacZ knock-in mice, researchers identify that no
lacZ-positive cell resides in the side population position with Hoechst staining (Asakura
et al., 2002; Beauchamp et al., 2000). In addition, Pax7 knockout mice display complete
absence of satellite cells in skeletal muscle, whereas the portion of SP cells are
unaffected, which give rise to 10-fold more hematopoietic colonies than myogenic
colonies (Seale et al., 2000). Furthermore, chick-mouse double labeling experiment
shows the origins of satellite cells and SP cells are traced during embryonic
development, which shows limb muscle SP cells exhibit heterogeneous origins co-
related to their functions. Some SP cells derive from the hypaxial somite as most
satellite cells do, which are intrinsically more myogenic than the nonsomatically derived
SP cells (Schienda et al., 2006). Taken together, these results demonstrate that SP
cells are not identical as satellite cells. Some of the SP cells are derived from myogenic
lineage and the others are derived from hematopoietic stem cells.

**Late-preplate (LP) cells**

Late-preplate (LP) cells from skeletal muscle exhibit multipotent stem cell
characteristics (Lee et al., 2000; Qu et al., 1998). LP cells are able to differentiate to
myogenic and osteogenic lineages both in vitro and in vivo (Lee et al., 2000). Torrente et al. have isolated and purified LP cells from skeletal muscle of mice through serial passage and FACS. The LP MDSCs exhibit positive expression of CD34 and Sca-1, and negative expression of desmin (Torrente et al., 2001). The transplantation of the enriched LP MDSCs into skeletal muscle displays a multipotential differentiation capacity, giving rise to skeletal muscle, glial cells and endothelial cell in vivo (Jankowski et al., 2002; Qu-Petersen et al., 2002). Moreover, LP MDSCs preserve their regeneration and self-renewal capability after long-term proliferation, which suggests that this population has the potential to improve muscle transplantation therapy (Deasy et al., 2005; Qu-Petersen et al., 2002). In summary, LP cells and SP cells are both multipotent stem cells located within skeletal muscle, which are different population from satellite cells. However, there is no clear evidence demonstrating the relationship between SP cells and LP cells.

**Other Stem Cells**

In addition to satellite cells and MDSCs, there are other stem cells located outside the basal lamina of muscle fibers, including pericytes, endothelial cells, and interstitial cells, all of which have been shown to have some myogenic potential. By contrast, some cells, such as bone marrow cells and hematopoietic cells, express some satellite cell markers but display no intrinsic myogenic capacity (Sherwood et al., 2004).

Mesoangioblasts are a class of vessel-associated fetal stem cells that can differentiate to most mesodermal cell types. They possess a high myogenic potential and contribute to muscle regeneration. Intra-arterial delivery of mesoangioblasts into dystrophic muscle of mice or dogs restores dystrophin expression, resulting in
restoration of functional muscle fibers and increase of motility (Sampaolesi et al., 2003; Sampaolesi et al., 2006).

Pericytes represent the adult vessel-associated stem cells, which associate with micro vascular wall in adult skeletal muscle. Pericytes from blood vessels of human muscle can regenerate and express myogenic markers only in differentiated myotubes with high efficiency. Importantly, when intra-arterial injected into dystrophic mice, pericyte-derived cells differentiate into muscle fibers more efficiently than satellite cells, probably because satellite cells can not cross the endothelial barrier. Moreover, small portions of pericyte-derived cells are located beneath the basal lamina of muscle fiber and express the satellite cell marker M-cadherin. Long-term cultures of these cells possess the intrinsic ability to respond to myogenic induction signals (Dellavalle et al., 2007).

CD133 (AC133) is a well-characterized marker of hematopoietic stem cells. A subpopulation of circulating cells express CD133 and some myogenic markers. These cells are able to migrate through the blood vessel in response to vascular cell adhesion molecular-1 (VCAM-1), and undergo myogenesis (Gavina et al., 2006). When these stem cells are transplanted into mdx mice intra-arterially or directly into the muscle, they repair the damaged muscle and recover the force production (Torrente et al., 2004). The regenerated cells are located under the basal lamina of host muscle fiber and express M-cadherin and Myf5. CD133+ stem cells isolated from blood and muscle of DMD patients can be engineered ex vivo to restore dystrophin expression and subsequently be delivered into dystrophic mouse muscle. These autologous donor cells
induce a significant recovery of muscle morphology, function, and dystrophin expression in dystrophic mice (Benchaouir et al., 2007).

PW1$^+$/Pax7$^-$ interstitial cells (PICs) are identified and purified from interstitium of skeletal muscle. They are myogenic in vitro, as well as efficiently contribute to skeletal muscle regeneration and repopulating satellite cells and PICs in vivo (Mitchell et al., 2010).

**Satellite Cell Niche**

Function of satellite cells is regulated by signals from the niche, microenvironment where satellite cell located. Satellite cells interact with the underlying myofiber via cell adhesion proteins, including M-cadherin and N-CAM (Irintchev et al., 1994). Satellite cells also communicate with the fiber via integrin α7β1 (Yao et al., 1996). In addition, satellite cells in the niche are exposed to several diffusible growth factors, which are required for their activity. Thus, the microenvironment of satellite cell niches, rather than satellite cells themselves, appears to be the key modulator of satellite cell activity. The regenerative capacity of satellite cells from aged mice is enhanced following exposure to young systemic factors (parabiosis), or culturing in the presence of young serum (Carlson and Conboy, 2007; Conboy et al., 2005). Rejuvenation is related to increased expression of the Notch ligand, Delta, and restoration of proliferation and regenerative capacity of satellite cells. In order to maintain the satellite cells niche, satellite cells are isolated and cultured with the associated muscle fiber in vitro (Bischoff, 1975; Zammit et al., 2004). However, the environmental factors that modulate satellite cell activity are still unclear.
Extracellular Matrix (ECM)

The ECM consists of fibrous protein (including laminin, tenascin, collagen, fibronectin and vitronectin) embedded in a mixture of proteoglycans. Satellite cell proliferation, migration and differentiation are regulated partially through interaction with different ECM molecules (Cornelison, 2008). Proteoglycans contain a core protein such as heparin sulfate (HS), chondroitin sulfate, dermatan sulfate, and keratin sulfate. The core protein links with several covalently attached glycosaminoglycan (GAG). Proteoglycans interact with growth factors and receptors through sulfate chains, which is required for growth factor-regulated satellite cell activity and muscle regeneration (Langsdorf et al., 2007; Riquelme et al., 2001; Villena and Brandan, 2004).

Extracellular matrix proteins have variable effects on satellite cell activity (Cornelison, 2008). For example, the basal side of the satellite cell expresses α7β1-integrin, which links satellite cells to the cytoskeleton with laminin within ECM. The linkage plays an essential role in transduction of mechanical forces to chemical signals, which is involved in regulation of satellite cell myogenesis (Boppart et al., 2006; Burkin and Kaufman, 1999). The apical side of satellite cells expresses M-cadherin, which attaches satellite cells to adjacent fibers, and stimulates myoblast fusion during muscle regeneration (Cornelison and Wold, 1997; Irintchev et al., 1994).

The ECM modulates activation of various signal transduction pathways, which are critical for satellite cell activities (Rhoads et al., 2009). One important function of ECM protein is to form complexes with fibroblast growth factor 2 (FGF2) and TGF-β (Velleman et al., 2004; Yamada et al., 1989). Recent studies attempted to improve the growth rate or regeneration efficiency of satellite cells by optimizing their culture system to include combinations of different ECM proteins, 3D matrix scaffold, and 3D matrix
associated with growth factors (Blumenthal et al., 2010; Carnio et al., 2010; Hill et al., 2006). Satellite cells cultured on Matrigel and laminin show increased integrin expression levels and exhibit an activated Wnt pathway (Wilschut et al., 2010). A three-dimensional collagen porous scaffold is used to deliver myogenic precursor cells to the muscular dystrophy mice. The muscle cells show decrease of apoptosis, increase of proliferation, and improvement of regeneration efficiency (Carnio et al., 2010). When satellite cells are delivered to the injured muscle with 3D matrix scaffold with sustained delivery of growth factors, such as HGF, FGF2, and VEGF-A, the long-term survival and migration of satellite cells are dramatically improved (Blumenthal et al., 2010; Hill et al., 2006).

**Wnt**

Wnt signaling is the major signal critical for both embryonic muscle development and postnatal muscle repair. Soluble Wnt ligands interact with Frizzled receptor, which associates with the lipoprotein receptor-related protein co-receptor (LRP). The coordination represses the axin/GSK3β/APC complex, which normally stimulates the degradation of β-catenin via ubiquitin pathway (Church and Francis-West, 2002).

During skeletal muscle development, surface ectoderm produces Wnt ligands and their effectors, which activate different myogenic regulatory factors (Fan et al., 1997). Canonical Wnt signaling directly activates Myf5 in the somite (Borello et al., 2006). Non-canonical Wnt signaling promotes the transcriptional activity of Pax3, which leads to expression of MyoD (Brunelli et al., 2007). Wnt ligands are also potent morphogens, and Wnt/b-catenin signaling is implicated in embryonic morphogenesis and regulation of adult stem cell function in many systems (Kleber and Sommer, 2004).
Similar to other growth factors, Wnt signaling also plays an important role in muscle repair and regeneration through regulating satellite cell fate (Brack et al., 2008; Cossu and Borello, 1999; Polesskaya et al., 2003). Wnt signaling is sufficient to convert CD45+/Sca1+ stem cells into the myogenic program during muscle regeneration (Polesskaya et al., 2003). By contrast, elevated Wnt signaling in the aged muscle seems to have an inhibitory role in maintenance of satellite cell identity and myogenic differentiation by promoting tissue fibrosis (Brack et al., 2007). Premature myogenic differentiation occurs in injured muscle injected with Wnt 3a (Brack et al., 2008). When proliferation C2C12 myoblasts or single fibers are treated with Wnt antagonists or secreted Frizzled receptor, the myoblasts or single fiber with either treatment show repressed myotube formation, decreased myogenin and MRF4 transcription, as well as decreased desmin expression (Brack et al., 2008). Moreover, there is impaired myotube formation along with decreased myogenin and MHC expression in BCL9 (a Wnt co-activator) knockout mice (Brack et al., 2009). If brief, Wnt signal switches satellite cell fate from differentiation to self-renewal.

**Notch**

Notch signal is activated when the membrane-anchored ligand (Delta/Jagged in vertebrates, LAG-2/APX-1 in C. elegans) interacts with the transmembrane Notch receptor, which then undergoes a series of cleavages resulting in the intracellular domain of Notch (NICD) translocation to the nucleus. NICD acts as a transcription factor that regulates target genes such as Hey and Hes, which encode proteins involved in myogenesis (rtavanis-Tsakonas et al., 1999).

Notch signaling is another important factor during muscle development and muscle injury. There is an increased expression of Notch signaling components (Delta-
1, Notch-1 and NlCD) in satellite cells and neighboring muscle fibers following muscle injury (Conboy et al., 2003; Conboy and Rando, 2002). Activation of Notch signal promotes satellite cell proliferation and prevents myoblast differentiation. When Notch signal is inhibited by Notch antagonist, Numb, γ-secretase inhibitor, or siRNA for presenilin-1, myoblast proliferation decreases and differentiation increases (Conboy and Rando, 2002; Dahlqvist et al., 2003; Kitzmann et al., 2006; Ono et al., 2009). By contrast, augment of Notch signaling through deletion of Stra13, a Notch inhibitor, compromises satellite cell differentiation and therefore muscle regeneration (Sun et al., 2007). Furthermore, Notch signaling promotes satellite cell quiescent and regulates satellite cell fate. Conditional knockout of Delta 1 (Notch ligand) or RBJ-J (transcriptional mediator of Notch) promotes myogenic differentiation, but depletes Pax3/Pax7-positive muscle progenitors, which leads to a failure of skeletal muscle growth (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Notch-3 is highly expressed in the quiescent satellite cells, particularly in the Myf5− stem cell subpopulation, whereas the Notch ligand Delta1 is highly expressed in the Myf5+ satellite cells (Fukada et al., 2007; Kuang et al., 2007). During muscle injury, Notch-1 and its antagonist Numb are expressed, which promote proliferation of satellite cells. During satellite cell division, Numb locates asymmetrically in active dividing cells, suggesting that Notch signal regulates satellite cell activation and fate determination (Conboy et al., 2003; Conboy and Rando, 2002).

During adult myogenesis, precise temporal Wnt and Notch signals cooperate to regulate muscle repair. Initially, Notch promotes proliferation of satellite cells. Wnt subsequently ensures committed satellite cells proceed to terminal differentiation.
GSK3β, an intracellular signaling component, plays a crucial role in switching from one pathway to another (Brack et al., 2008).

**Myostatin**

Myostatin, also called growth and differentiation factor 8 (GDF8), is a member of TGF-β superfamily. It is the major limiting factor of muscle size and negative regulator of skeletal muscle growth and development (Solomon and Bouloux, 2006). Naturally deficient or genetically mutation of functional myostatin in cattle or mice leads to an increase in skeletal muscle mass, which is referred as double-muscling phenotype (Lee, 2007; McPherron et al., 1997). The molecular basis of the double-muscling phenotype is muscle hyperplasia (increases in muscle fiber number) instead of muscle hypertrophy (increases in muscle fiber size). This is different from normal pattern of postnatal muscle growth (McPherron et al., 1997). Recent studies indicate that myostatin expressed by satellite cells plays a role in repression of satellite cell proliferation and enhancement of satellite cell self-renewal (McCroskery et al., 2003; McFarlane et al., 2008). Studies of chick embryo also show that myostatin affects the balance between proliferation and differentiation of myogenic precursor cells (Pax7+/Myf5+) by promoting cell cycle withdrawal and muscle differentiation (Manceau et al., 2008). Thus, myostatin negatively regulates satellite cell function through withdrawal of satellite cells from cell cycle, and leading them either to a quiescent state or a terminal differentiation state.

**IGF-I**

The IGF system contains two ligands (IGF-I and IGF-II), three receptors, and six IGF binding proteins (IGFBP1-6). Compared with IGF-II, IGF-I plays a primary role in the regulation of satellite cell activity (Florini et al., 1996; Liu et al., 1993). Mice lacking IGF-I lose 60% body weight as well as exhibit muscle lost and developmental
retardance (Liu et al., 1993; Powell-Braxton et al., 1993). Transgenic mice with overexpression of IGF-I have a 30% raise in body weight due to increase in skeletal muscle and bone (Mathews et al., 1988). Local expression of active IGF-I isoform in skeletal muscle also leads to muscle hypertrophy, which protects the transgenic mice from age-induced muscle lost (Musaro et al., 2001). These results suggest that IGF-I is important for skeletal muscle growth and development. Furthermore, IGF-II(-/-) mice also lose 40% body weight, with more serious phenotype exhibited in IGF-IR, IGF-I/IGF-IR, IGF-II/IGF-IR, IGF-I/IGF-II knockout mice, which demonstrates that components of IGF system interact with each other and cooperate to regulate body size and muscle growth (Liu et al., 1993).

In skeletal muscle, there are at least two distinct IGF-I isoforms generated by alternative splicing of the gene: the systemic form (IGF-1Ea), which is released form liver in a growth hormone (GH)-dependent manner; and mechano growth factor (MGF), which is locally produced by skeletal muscle in response to muscle contraction (Goldspink, 2007; Yang et al., 1996). As a result, IGF-I regulates skeletal muscle and satellite cells through endocrine, paracrine, and autocrine manners.

IGF-I is able to stimulate satellite cell proliferation and differentiation depending on the developmental stages (Dodson et al., 1996). To balance the mitogenic and myogenic actions on skeletal muscle, IGF-I has a biphasic effect on satellite cells. Initially, IGF-I inhibits expression of myogenin, which results in a proliferation response. Subsequently, IGF-I switches to stimulate myogenin expression, which changes satellite cells from proliferation to differentiation (Coolican et al., 1997). The diverse actions of IGF-I are mediated through numerous intracellular signaling cascades. IGF-I binds to
IGF-IR, which causes phosphorylation and activation of insulin receptor substrates (IRSs). The phosphorylated IRSs then recruit some Src homology 2 (SH2) domain containing scaffold adaptors, such as growth factor receptor binding-2 protein (Grb2) and p85 regulatory subunit of the phosphatidylinositol 3' kinase (PI3K) (Giovannini et al., 2008). By binding to Grb2, IGF-I signaling activates Ras-MEK-ERK pathway, which regulates DNA synthesis and satellite cell proliferation. By binding to p85 subunit of PI3K, IGF-I signaling activates PI3K-Akt pathway, which regulates protein synthesis and satellite cell differentiation (Dupont et al., 2003).

**FGF2**

IGF-I and FGF2 are the most extensively studied positive regulators of skeletal muscle. FGF2 is located in the extracellular matrix of skeletal muscle fibers and bound to the basal lamina. The accumulation of FGF2 appears to augment in hypertrophy muscle fibers (Yamada et al., 1989). FGF2 is also required for proliferation of both avian embryonic myoblast and adult avian satellite cells (McFarland et al., 1993). In vivo, FGF2 promotes satellite cell proliferation and muscle regeneration in mice (Lefaucheur and Sebille, 1995). Moreover, cooperation of FGF2 with other muscle regulators (HGF, VEGF-A, SDF-1, Akt) in extracellular matrix exhibits an enhancement of myoblast transplantation efficiency. The enhancement is due to improved cell survival, increased cell proliferation and migration (Blumenthal et al., 2010; Hill et al., 2006).

FGF family contains more than 20 ligands and 4 receptors. The ligands (FGF1, 2, 4, 5, 6, 8, 10) and the receptors (FGFR1, 2, 4) are expressed in skeletal muscle (Dodson et al., 1996; Sheehan and Allen, 1999). Other components of FGF signaling also play an important role in regulation of skeletal muscle and satellite cells. For example, FGF1, 2, 4, 6 and 9 stimulate proliferation of rat satellite cells (Sheehan and
Allen, 1999). Blocking FGF signal through overexpression of a dominant negative FGF receptor 1 causes reduction of skeletal muscle mass, disruption of muscle organization, and interruption of limb development. These actions are due to suppression of myogenic lineage, inhibition of satellite cell proliferation, and enhancement of myoblasts differentiation (Flanagan-Steet et al., 2000; Scata et al., 1999). Sprouty, a FGF signal antagonist, modulates the balance between self-renewal and differentiation of Pax3/7-positive progenitor cells (Lagha et al., 2008). Moreover, Heparin sulfate (HS), an ECM component that promotes FGF-FGFR dimerization, is required for muscle regeneration and affects FGF signaling during the transition from satellite cell proliferation to differentiation (Langsdorf et al., 2007; Mohammadi et al., 2005).

FGFRs are members of receptor tyrosine kinase (RTK) family, whose autophosphorylation leads to a series of downstream phosphorylation cascades. Among the intracellular pathways, Ras-MEK-MAPK pathway is essential for FGF-induced regulation of satellite cell proliferation and differentiation (McFarland and Pesall, 2008; Weyman and Wolfman, 1998). Moreover, ERK2 is essential for FGF2-induced myoblast differentiation, but ERK1 is dispensable for FGF2 signaling in myoblasts (Li and Johnson, 2006).

**HGF**

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is originally identified by its ability to induce hepatocyte growth (Bottaro et al., 1991), or to scatter epithelial cells (Weidner et al., 1990). In skeletal muscle, HGF is present in the extracellular matrix of the muscle and its receptor c-met is localized at the surface of satellite cells and adjacent myofibers (Hawke and Garry, 2001).
HGF and its receptor c-met play an important role in skeletal muscle development. Delamination and migration of muscle progenitor cells depend on the presence of HGF receptor c-met. HGF, produced by the mesenchymal cells of the limb, provides the positive cues and delineates the migration route for the muscle progenitor cells (Dietrich et al., 1999). In addition, in mutant mouse embryos lacking functional c-met (Bladt et al., 1995) or HGF (Schmidt et al., 1995), skeletal muscle is absent from developing limbs.

HGF has been well studied as an activator of satellite cells. It is released from ECM upon muscle injury through a nitric oxide (NO)-dependent manner (Allen et al., 1995; Sheehan et al., 2000; Tatsumi et al., 1998). Muscle injury causes nitric oxide (NO) to release, which is activated by nitric oxide synthase (NOS), and in turn activates matrix metalloproteinase (MMP) (Tatsumi et al., 2002; Tatsumi et al., 2006). MMP is capable of degrading several ECM proteins and promoting HGF release from the ECM (Yamada et al., 2008; Yamada et al., 2006). Besides activating quiescent satellite cells, HGF is also involved in regulating multiple cellular processes in adult skeletal muscle. It stimulates satellite cell proliferation by increasing DNA synthesis, and inhibits satellite cell differentiation by suppressing the activity of basic helix-loop-helix (bHLH)/E-protein complex (Gal-Levi et al., 1998). Satellite cells migration is also promoted by HGF though degrading of cell surface glycosaminoglycans (GAGs) (Bischoff, 1997; Villena and Brandan, 2004). Moreover, HGF triggers satellite cell activation and up-regulates neural chemorepellent Sema3A, suggesting that HGF is required for interaction between motor innervation and satellite cell activation to maintain the postnatal myogenesis (Tatsumi et al., 2009).
Since c-met is the only receptor for HGF, the multiple cellular responses induced by HGF seem to be mediated though different pathways downstream of c-met. It is reported that binding of intracellular mediator Grb2 to activated c-met induces activation of Ras-Raf-MAPK cascade and in turn stimulates satellite cell activation (Leshem et al., 2002). By contrast, c-met activates the PI3K/Akt pathway through coupling Gab1 adaptor, which results in inhibition of satellite cell differentiation (Halevy and Cantley, 2004).

**Ephrin**

Ephrin ligands associate with the plasma membrane either by a glycosylphosphatidylinositol (GPI) anchor (ephrinA1-A5) or by a transmembrane domain (ephrinB1-B3). Eph receptor tyrosine kinases (RTKs) family constitutes the largest class of RTKs, with at least 14 members identified. Eph RTKs are separated into two sub-classes: EphA and EphB, depending on which types of ligands they interact with. Ephrin and Eph receptor interaction transduces bidirectional signaling, which triggers both the receptor-expressing cell (forward signaling) and the ligand-expressing cell (reverse signaling) [reviewed in (Kuijper et al., 2007)].

Ephrin-Eph RTK system displays an essential role in guidance of motor neuron and regulation of cell migration during embryonic development (Egea and Klein, 2007). For example, EphA3 and EphA4 are found to express in either medial motor column or branchial and lumbar segments of spinal cord, which innervate axial muscle or limb muscle respectively (Kilpatrick et al., 1996; Ohta et al., 1996). Varies Eph RTKs and ligands, such as ephrin-A2, ephrin-A5, EphA3, EphA4, and EphA5, are co-expressed in developing muscles, as well as in the motor neurons innervate them and their surrounding connective tissue (Iwamasa et al., 1999; Tono-Oka et al., 1996). The
expression pattern of ephrin system members suggests that they are required for muscle patterning and innervation.

Some ephrin receptors, such as EphA4 and EphA7, are continuously expressed through fetal development until adulthood, indicating they have additional functions in adult muscle (Lai et al., 1999; Lai et al., 2001). Interestingly, EphA4 tends expressed in the postsynaptic apparatus of muscle, from postnatal day 7 to day 21, which is a similar time schedule as neuromuscular junction (NMJ) maturation (Bewick et al., 1996). In addition, EphA4 and EphA7 are highly expressed in adult NMJ, and their expression is regulated through a nerve-dependent manner (Lai et al., 2001). Moreover, the EphA4 knockout mice move their hindlimbs simultaneously rather than in an alternating manner due to impaired motor control (Kullander et al., 2001). These results all support the idea that EphA4 is crucial for formation and maintenance of neuromuscular junction (NMJ), through guiding axon into skeletal muscle and maintaining muscle innervation (Lai and Ip, 2003).

Ephrin-Eph RTK signal regulates migration and attachment of different types of cells in two opposite manners, either as a chemo-attractive cue or as a chemo-repulsive cue. Ephrin signal has been well defined as a chemorepellent for neural axon guidance (Klein, 2004). For example, activation of Eph receptor of migrating neural crest cells triggers retraction away from ephrin-expressing tissues (Klein, 2004). However, ephrin is also reported as an attractive or adhesive factor in many cell types, including neural cell (Stein et al., 1998), endothelial cell (Holmberg et al., 2000), and neuroepithelial cell (Hansen et al., 2004). For example, low concentration of ephrin-A2 promotes cultured nerve cell adhesion (Hansen et al., 2004).
During avian skeletal muscle development, ephrin-A5 is expressed in the forelimb mesoderm, and it interacts with EphA4 on the surface of Pax7-positive muscle precursors, through which ephrin-A5 restricts the migration of muscle precursors and leads them to their appropriate territories in forelimb (Swartz et al., 2001). In adult skeletal muscle, EphA4 locates at the NMJ region, where satellite cells have also been found (Lai et al., 2001; Powell et al., 2003). The same location raises the possibility that ephrin-Eph receptor interaction may relate to satellite cell migration and localization in adult muscle.

Ephrin regulates cell migration through various intracellular signal pathways. Activated Eph RTKs are able to interact with a series of downstream proteins, including phosphotyrosine adaptors (Grb2, Gab1, Crk, Nck), PDZ domain proteins, PI3K subunit, as well as modulators of Ras and Rho GTPases (Lai and Ip, 2003). Rho GTPases are considered as the major signal involved in ephrin-induced migration (Schmitz et al., 2000; Wahl et al., 2000). The Rho family small GTPase has three members, Rac1, Cdc42 and RhoA, which cause diverse cellular effects (Kozma et al., 1997). Eph RTKs modulate the activities of different Rho GTPases via GTPase-activating protein (GAP) or Guanine nucleotide exchange factor (GEF), such as Ras GAP (Holland et al., 1997) and ephexin (Shamah et al., 2001). Ephrin-Eph RTK-Rho GTPase signal regulates cell migration via rearrangement of actin cytoskeleton (Lai et al., 2001; Meima et al., 1997).
CHAPTER 2
CENTRAL HYPOTHESIS

Satellite cell is the major cell type responsible for skeletal muscle growth and hypertrophy. In order to clarify the molecular mechanism of postnatal muscle growth of meat-producing animals, the heterogeneity and activity of satellite cells need to be understood. Some growth factors play an essential role in regulating satellite cell identification, proliferation, migration, and differentiation.

My central hypothesis is that primary bovine satellite cells can be used as a model to study satellite cell activity in meat-producing animals, which is regulated through both intrinsic cell identity and extrinsic growth factors. Assessment of bovine satellite cell identity and activity reveals the cellular and molecular mechanism of growth regulation in meat animal, and provide the possibility of increasing the efficiency of lean meat production. The following objectives were designed to test the hypothesis:

- **Objective 1:** Evaluate the functions of HGF and SHP2 signals during proliferation of myoblasts and mouse satellite cells.
- **Objective 2:** Characterize the heterogeneity and myogenesis of bovine satellite cells.
- **Objective 3:** Study the effects of ephrin-A5 on migration of myoblasts and bovine satellite cells.
Satellite cells are the resident stem cells of postnatal skeletal muscle tissue (Dhawan and Rando, 2005). In adults, these cells are located adjacent to the muscle fiber in a quiescent state and become mitotically active in response to tissue growth and repair stimuli (Wozniak et al., 2005). Upon reentry into G\textsubscript{1}, satellite cells proliferate and differentiate in a manner analogous to their embryonic myoblast counterparts. The transition period between G\textsubscript{0} and G\textsubscript{1} of the cell cycle, referred to as activation, is regulated by locally produced growth factors. Niche factors participating in activation include Notch ligands, nitric oxide and hepatocyte growth factor (HGF) (Allen et al., 1995; Anderson, 2000; Conboy and Rando, 2002; Miller et al., 2000; Tatsumi et al., 1998; Wozniak and Anderson, 2007). Negative mediators of activation remain poorly defined but may include growth and differentiation factor 8 (GDF8) (McCroskery et al., 2003).

Initial experiments defining the activation period of satellite cells revealed the existence of a protein in crude preparations of crushed skeletal muscle that caused fiber-associated satellite cells to emerge from the quiescent state (Bischoff, 1986). The requisite factor proved to be HGF, a mitogen for several cell types (Tatsumi et al., 1998). Treatment of satellite cells with HGF stimulated activation and subsequent proliferation in vitro (Sheehan et al., 2000). Suppression of HGF activity with an antibody prevents autocrine HGF-mediated activation. Injection of HGF into sites of damaged skeletal muscle causes an increase in the numbers of mitotically active satellite cells within the lesion (Miller et al., 2000). And, the growth factor is released from the fibers during periods of stretch exercise (Tatsumi et al., 2001; Tatsumi et al.,
HGF initiates signals via docking with its transmembrane localized, tyrosine kinase receptor, MET (Rosario and Birchmeier, 2003). MET is critical for normal limb muscle formation as it denotes the migratory myoblast population (Bladt et al., 1995). Mice genetically devoid of MET display normal specification of myoblasts but the cells remain at the proximal limb border failing to migrate and populate the muscle beds. Gab1, an adaptor protein with no inherent kinase activity, orchestrates the intracellular signals underlying the migratory response. Gab1 recruitment to the MET kinase domain is necessary for emigration of myoblasts from the dermomyotome (Sachs et al., 2000). Mice lacking Gab1 contain fewer and smaller muscles within both the fore and hind limbs. The docking protein mediates the migratory actions of myoblasts by interaction with SHP2, a protein tyrosine phosphatase. Disruption of Gab1:SHP2 interactions duplicates the defective migratory patterns found in mice devoid of MET or Gab1.

The role of SHP2 during myogenesis remains poorly defined. Genetic ablation of SHP2 results in mice with mesoderm defects prior to specification of the myogenic cell populations (Saxton et al., 1997). Conditional ablation of SHP2 in nestin-expressing (nestin$^{SHP2(-/-)}$) cells causes neural defects that result in early neonatal lethality (Ke et al., 2007). Interestingly, nestin$^{SHP2(-/-)}$ mice exhibit a reduction in muscle mass that phenotypically resembles that of Pax7$^{(-/-)}$ mice (Seale et al., 2000). Nestin is expressed in quiescent satellite cells thus, suggesting that the phosphatase may participate in aspects of satellite cell biology (Day et al., 2007). The objective of these experiments was to examine SHP2 expression in satellite cells and provide insight into the
importance of the protein during myogenesis. Results presented herein demonstrate that SHP2 limits satellite cell proliferation with no apparent effects on differentiation. Knockdown of SHP2 increases the number of cycling cells independent of ERK1/2 actions. Importantly, SHP2 is a key intracellular mediator of the repressive actions of HGF on satellite cell cycle transit. Elevated concentrations of HGF cause cell cycle exit that are overcome in the absence of SHP2. We conclude that HGF both positively and negatively effects activation in satellite cells and its inhibitory activities are mediated through SHP2.

**Materials and Methods**

**Satellite Cell Isolation and Cell Culture**

Adult Balb-C female mice were euthanized by CO₂ inhalation followed by cervical dislocation according to the University of Florida Institutional Animal Care and Use Committee guidelines. Hindlimb muscle groups were harvested from adult mice and connective tissue was removed. The tissue was finely minced and digested with proteinase E (1.5 mg/ml in sterile phosphate buffered saline [PBS]) for 60 minutes at 37°C. The proteinase was removed by centrifugation of the tissue slurry at 800 X G for 10 minutes. An equal volume of PBS was added to the tissue, vortexed for 5 minutes and centrifuged at 400 X G for 10 minutes. The process was repeated for a total of four times with the supernatants retained after each centrifugation. The final cell slurry was passaged sequentially through 70 μm and 40 μm sieves to remove debris. The cells were collected by centrifugation and seeded in tissue cultureware or stored frozen in liquid nitrogen.

Satellite cells were routinely cultured in high glucose Dulbecco’s Eagle medium supplemented with 10% horse serum, 1% penicillin-streptomycin and 0.5% gentamicin
on entactin/collagen/laminin-coated plates (ECL; Invitrogen). 23A2 embryonic mouse myoblasts were cultured on gelatin-coated tissue cultureware in basal Eagle medium supplemented with 15% fetal bovine serum, 1% antibiotic and 0.2% gentamicin. Stable cell lines (23A2-SHP2-WT, -SHP2-CA, -SHP2-DN) were cultured in growth media supplemented with 400 μg/ml geneticin (G418, Invitrogen). Differentiation of myoblasts was induced by culture in low glucose DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin.

**Plasmids and Cell Transfection**

Mammalian expression plasmids contained the cDNA inserts SHP2-WT, coding for wild-type mouse SHP2, SHP2-CA, coding for mouse SHP2 with a substitution at glutamic acid 76 to alanine (E76A) creating a constitutive active phosphatase and SHP2-DN, coding for a catalytically inactive mutant of SHP2 created by substitution of cysteine 459 with serine (C463S). The mutant SHP2 cDNAs were created by PCR-mediated mutagenesis using wild-type mouse SHP2 cDNA as the template and AccuPrime DNA polymerase. DNA sequence of both strands was verified. cDNA inserts were cloned in frame with a multimerized FLAG epitope to create CMV-SHP2 plasmids. Double-stranded DNA coding for a small interfering RNA to SHP2 was created with 5-CAGGAGCTGAAATACGACG, corresponding to mouse SHP2 nt637-655, and 5-CCCAAAAAGAGTTACATTG, corresponding to nt1080-1101 of mouse SHP2 mRNA, and the complimentary DNA. The double stranded cDNAs were inserted into pSIREN-RetroQZsGreen (Clontech Laboratories) for siRNA transcription from the human U6. All plasmids were purified using Qiagen Maxi purification columns followed by dialysis against 10 mM TRIS, pH 8.0. Myogenic cells were transiently transfected in growth media using FuGene6 (Roche) with 3 volumes reagent and 2 μg plasmid DNA.
Western Blot

Total cell lysates were harvested by direct lyses in SDS-PAGE sample buffer. Protein lysates representing an equivalent number of cells were separated through 10% polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked for 30 minutes with 10% nonfat dry milk in TRIS-buffered saline containing 0.1% Tween20 (TBST). Subsequently, blots were incubated with primary antibodies diluted in 1% nonfat dry milk in TBST. Antibodies included anti-tubulin (1:5000; Abcam), anti-ERK1/2 and antiphosphoERK1/2 (1:1000, Cell Signaling), anti-SHP2 and anti-phosphoSHP2 (1:1000, Cell Signaling), and anti-FLAG (1:200, M2, SigmaAldrich). Blots were washed extensively with TBST prior to incubation with the appropriate peroxidase-labeled secondary antibody. Immune complexes were visualized by chemiluminescence (ECL, Amersham) and autoradiography.

Immunocytochemistry

Cells were fixed with fresh 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized and nonspecific binding sites were blocked with 5% horse serum in PBS containing 0.1% Triton X100. Cells were further incubated overnight at 4 C in mouse anti-Pax7 (1:4 hybridoma supernatant, Developmental Studies Hybridoma Bank), mouse anti-PCNA (1:50, PC10, Santa Cruz Biotech), mouse anti-MyoD (1:30, 5.8A, Vector Laboratories), mouse anti-myosin heavy chain (1:5 hybridoma supernatant, MF20, Developmental Studies Hybridoma Bank) and rabbit anti-Ki67 (1:200, SP6, Abcam). After exhaustive washing with PBS, the cells were incubated with goat anti-mouse AlexaFluor488 or AlexaFluor568 (1:200, Invitrogen) or donkey anti-rabbit AlexaFluor488 or AlexaFluor568 (1:200, Invitrogen) for 40 minutes at room temperature. Total nuclei were detected with Hoechst 33342 (10 μg/ml in PBS).
Immune complexes were visualized with a Nikon TE2000 inverted phase microscope equipped with epifluorescence. Representative images were captured with a CoolPix CCD camera and assembled and analyzed with NIS-Elements software (Nikon).

**Statistical Analysis**

All data were analyzed by ANOVA followed by t-test using Statistical Analysis System (SAS Institute Inc., Cary, NC). Results were presented as the mean + SEM. A p-value <0.05 was considered to be significant.

**Results**

MET initiation of downstream Gab1:SHP2 signals is necessary for limb formation and myoblast migration during development (Sachs et al., 2000; Schaeper et al., 2007; Schaeper et al., 2000). Because MET signaling stimulates satellite cell activation and migration (Allen et al., 1995; Nishimura et al., 2008; Yamada et al., 2008), we examined the importance of SHP2 in mediating HGF effects on these cells. As a first step, stable 23A2 myoblast cell lines were created that over-express wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or a phosphatase defective SHP2 (SHP2-DN). 23A2 myoblasts were selected because they contain a high percentage (approx. 30%) of satellite cell progenitors (\(\text{Pax7}^+\cdot\text{MRF}^+\)) (Reed et al., 2007). Initial Western blot analysis reveals that all of the SHP2 cell lines express abundant amounts of the FLAG-tagged fusion phosphatase of the correct size (Figure 3-1). The proliferative effects of the mutant SHP2 proteins were evaluated by BrdU pulse labeling (Figure 3-2). In brief, the SHP2-expressing myoblasts were seeded at equal cell density in growth media. Two hours prior to fixation, the subconfluent cells were pulsed with BrdU for the immunodetection of proliferating cells. Approximately 30% of the 23A2 control cells are traversing S-phase when maintained as subconfluent monolayers in growth media. 23A2-
SHP2-DN incorporates BrdU at rates comparable to control cells suggesting that SHP2 is not required for proliferation. By contrast, both 23A2-SHP2-WT and 23A2-SHP2-CA exhibit an increased percentage of cells synthesizing DNA arguing that the phosphatase elicits a positive response on proliferation. The effects of sustained SHP2 activity on myofiber formation was examined (Figure 3-3). Confluent 23A2-SHP2 expressing myoblasts were cultured for 48 h in differentiation permissive media. In all instances, large multinucleated myofibers formed that express the contractile protein, myosin heavy chain (MyHC). Induction of the full myogenic program was evaluated further by Western analysis for myogenin, the requisite MRF for differentiation. Densitometric analysis of the blots reveals that neither SHP2-WT nor SHP2-DN affects myogenin protein production (Figure 3-4). Interestingly, 23A2-SHP2-CA demonstrates a substantial reduction in the amounts of myogenin protein. From these results, we conclude that SHP2 may participate in myoblast proliferation and elicits differential effects on myoblast differentiation.

HGF stimulates satellite cell proliferation in vitro (Allen et al., 1995; Gal-Levi et al., 1998; Sheehan et al., 2000; Tatsumi et al., 2002). The interplay between SHP2 and HGF was examined in the mutant cell lines. 23A2, 23A2-SHP-WT, -SHP2-CA and -SHP-DN myoblasts were culture in reduced serum media supplemented with HGF for 48 h. The cells were treated with BrdU for 2 h prior to fixation and immunodetection of the thymidine analog. As expected, HGF stimulates myoblast proliferation as evidenced by an increase in the percentage of cell progressing through S-phase (Figure 3-5). A higher percentage of 23A2-SHP2-WT and 23A2-SHP2-CA myoblasts are proliferating than controls and this number is not further improved by HGF treatment. 23A2-SHP2-DN myoblasts divide at rates comparable to parental 23A2 myoblasts. However,
treatment with HGF does not improve proliferation. These results indicate that SHP2 is the major mediator of HGF-induced myoblast proliferation.

SHP2 prolongs the activity of downstream ERK1/2 (Mohi and Neel, 2007; Neel et al., 2003). The requirement of ERK1/2 in the SHP2 response was further explored in 23A2-SHP2-WT myoblasts. In brief, 23A2 and 23A2-SHP-WT myoblasts were cultured in low-serum media supplemented with HGF and PD98059, a chemical inhibitor of MEK1/2, or an equivalent volume of vehicle-only. After 48 h, proliferation was assessed by BrdU incorporation as described throughout. As expected, more mitotically active myoblasts are present in the 23A2-SHP2-WT cultures (Figure 3-7). Treatment with the MEK inhibitor causes a reduction in the percentage of dividing SHP2-expressing myoblasts. Supplementation of the culture media with HGF does not affect 23A2-SHP2-WT proliferation rates. Importantly, inhibition of MEK/ERK activity in conjunction with HGF treatment has no effect on 23A2-SHP2-WT proliferation. The ability of SHP2 to compensate for the loss of MEK/ERK signaling argues that the two signaling systems are independent of one another in myogenic cells. The ERK-independent actions of SHP2 are further supported by examination of the ERK1/2 activation profile in response to HGF treatment. Subconfluent 23A2, -SHP2-WT, -SHP2-CA and -SHP2-DN myoblasts were washed with protamine sulfate to remove ECM-bound growth factors and cultured for 60 min in serum-free medium. Subsequently, the cells were treated with HGF and lysed over time for assessment of ERK activity. Western blot for phosphorylated ERK1/2 demonstrates that all cells direct a robust response to HGF at 10 min and the amounts of phosphoERK1/2 are severely diminished, or completely absent, by 20 min post-stimulus (Figure 3-6A). Semi-quantification of the relative
amounts of phosphoERK1/2 demonstrates no differences in the kinase activity profile in response to HGF (Figure 3-6B).

The role of SHP2 was further evaluated in primary cultures of mouse satellite cells. Satellite cells were isolated from adult mice and cultured en masse for 3 days prior to transfection with plasmid encoding SHP2-WT. After 48 h, the cells were fixed and proliferating cells enumerated following immunocytochemical detection of PCNA in the SHP2 expressing cells. Results indicate that unlike 23A2 myoblasts, satellite cell mitotic activity is unaffected by ectopic SHP2 (Figure 3-8). Importantly, the phosphatase does not interfere with the identity of the satellite cells. No differences in the number of Pax7 or MyoD immunopositive cells were evident. These results argue that primary satellite cells are not equivalent to 23A2 myoblasts with regard to SHP2-mediated signaling events. The amount of activated and total SHP2 was further evaluated. Western blot showed that mouse satellite cells expressed equivalent amount of SHP2 protein as 23A2 myoblasts. However, the active forms of SHP2 were reduced in 23A2 myoblasts (Fig 3-9). To better understand the importance of SHP2 in primary satellite cells, the relative amounts of the phosphatase were reduced. Small interfering RNAs directed against SHP2 were created and evaluated for knockdown efficiency. 23A2 myoblasts were transiently transfected with pSilencer-siSHP2 or the scrambled control, pSilencer-siCon. Transfection efficiency exceeds 50% in these cells. After 48 h, total cell lysates were harvested and analyzed by Western for SHP2. As shown in Figure 3-10, 23A2 myoblasts express SHP2 and the endogenous phosphatase levels are not affected by the scrambled control siRNA. By contrast, ectopic expression of siSHP2 causes a substantial reduction in SHP2 protein content. No differences were observed in tubulin
levels indicating equivalent amounts of total protein and cell numbers. Subsequently, the mouse siSHP2 cDNA was cloned into a lentiviral vector that constitutively expresses GFP. Primary satellite cells were transiently transfected with the vector coding for siSHP2 or siCon. After 24 h the GFP-expressing cells were evaluated for the presence or absence of Ki67, a proliferation marker. Approximately 60% of the satellite cells expressing the scrambled control siRNA were mitotically active (Figure 3-11). Satellite cells ectopically expressing siSHP2 demonstrate a higher percentage of proliferating cells. Thus, it appears that SHP2 represents a putative block to optimal proliferation in satellite cells.

Strong ERK1/2 activity prevents proliferation in myoblasts and induces a reversible quiescent state (Reed et al., 2007; Wang et al., 2004). Because HGF can induce the MEK/ERK signaling axis in satellite cells (Halevy and Cantley, 2004), we examined the effects of the growth factor on cell cycle transition. Semiconfluent cultures of satellite cells were treated with 10 or 50 ng/ml HGF for 48 h in low-serum media. Two hours prior to termination, the cells were pulsed with BrdU. The numbers of BrdU-incorporating cells and total cell numbers were quantified. As expected, the lower concentration of HGF readily stimulated cell proliferation as indicated by an increase in the percentage of cells incorporating BrdU (Figure 3-12). The higher concentration of HGF caused a significant reduction in the numbers of satellite cells progressing through S-phase. The ability of high concentrations of HGF to induce ERK and SHP2 were evaluated by Western blot. Total cell lysates were harvested at 10-minute intervals following treatment with 50 ng/ml HGF (Figure 3-14). A robust increase in phosphoERK1/2 is evident within 10 min of HGF supplementation followed by a decline.
to basal activity within 30 min. By contrast, SHP2 is present in a phosphorylated form prior to HGF treatment and the amounts of phosphoSHP2 do not vary across the experimental time frame. No differences in total SHP2, ERK1/2 or tubulin content are apparent. Further study was carried out to understand the roles of SHP2 and ERK1/2 in the HGF-induced growth arrest. Satellite cells were treated with or without HGF and PD98059 for 48 hours prior to test for BrdU incorporation. Either blocking ERK1/2 with inhibitor or HGF treatment along caused a decreased proliferation rate. However, when the cells were treated with both HGF and PD98059, the HGF-induced growth arrest was partially restored (Figure 3-15). Thus, HGF represses satellite cell proliferation through both ERK1/2-dependent and ERK1/2-independent manner. These results demonstrate that SHP2 is present but does not appear to prolong the HGF-mediated ERK1/2 signal.

The role of SHP2 in the HGF-imposed block to cell cycle progression was further explored by knockdown of the phosphatase levels. Primary satellite cell cultures were transfected with a lentiviral vector constitutively expressing GFP and siCon or siSHP2. After 24 h, the cells were further incubated in low-serum media supplemented with 50 ng/ml of HGF or an equivalent volume of vehicle-only. GFP-expressing satellite cells were evaluated for Ki67 expression after 48 h of growth factor treatment. Knockdown of SHP2 results in an increase in the numbers of proliferating cells, as shown previously (Figure 3-13). Treatment with 50 ng/ml HGF did not reduce the percentage of proliferating cells by comparison to satellite cells transfected with the scrambled control. The numbers of proliferating siSHP2 cells remain unchanged in the presence of the inhibitory concentration of HGF suggesting that the phosphatase participates in the HGF-induced growth arrest of satellite cells.
Discussion

The importance of HGF for satellite cell activation is indisputable. However, the role of the growth factor during deactivation and the return to \( G_0 \) is novel and intriguing. HGF is an autocrine factor synthesized and released during the period of rapid proliferation and subsequent self-renewal of the population making it present at the correct time and location (Allen et al., 1995; Jennische et al., 1993; Tatsumi et al., 1998; Tatsumi and Allen, 2004). Injection of HGF into a site of muscle damage results in an increase in the numbers of MyoD-expressing satellite cells and a reduction in fiber formation (Miller et al., 2000). These results support widely documented in vitro evidence that HGF stimulates proliferation and inhibits differentiation. After 48 h in culture, satellite cells en mass are considered activated and fully proliferative (Beauchamp et al., 2000; Zammit et al., 2004). This is reflected in our results whereby a low-dose HGF stimulus causes a significant increase in the percentage of satellite cells incorporating the thymidine analog. By contrast, a higher concentration of HGF (50 ng/ml) diminishes satellite cell proliferation. The ability of HGF to alter satellite cell activity as a consequence of signal intensity is supportive of a dual role for the growth factor. In our model, autocrine HGF is present at low concentrations during the initial stages of skeletal muscle damage such that satellite cell activation and subsequent proliferation occur. As the numbers of active satellite cells increase, the local concentration of HGF becomes sufficient to induce cell cycle exit and a return to the quiescent state. This same model can be extrapolated to times of normal skeletal muscle growth and exercise-induced hypertrophy. In this scenario, the individual satellite cell responds to the autocrine factor to divide once with one cell returning to \( G_0 \) and the daughter fusing into the adjacent fiber. In addition to encouraging a \( G_0 \)-like
state, high concentrations of HGF may protect the satellite cell from precocious differentiation. Immunocytochemical detection of myogenin reveals no increase in the numbers of differentiated cells (data not shown) arguing that the HGF-imposed G_0 is not permissive to terminal differentiation. It remains to be determined if high HGF concentrations can elicit similar effects in the presence of a strong differentiation inducing signal such as that supplied by IGF-I.

SHP2 is a protein tyrosine phosphatase that is recruited to the MET kinase domain via the docking protein, Gab1 (Rosario and Birchmeier, 2003). Either direct or indirect interaction of Gab1:SHP2 is sufficient to promote myoblast migration into the developing limbs of mice (Sachs et al., 2000; Schaeper et al., 2007; Schaeper et al., 2000). The importance of SHP2 to satellite cell biology is not limited to migratory actions but appears to include cell cycle kinetics. Satellite cells in vitro express the protein and loss of SHP2 causes an increase in the numbers of proliferating cells. Ectopic expression of SHP2 also tends to decrease proliferation as indicated by a reduction in the numbers of PCNA expressing satellite cells. Of importance, loss of SHP2 prevents the inhibitory actions of high concentrations of HGF. These results support a model whereby HGF signals through SHP2 to inhibit proliferation and impose a quiescent program on the satellite cell. In many cell types, SHP2 activities include a prolonged ERK1/2 response (Mohi and Neel, 2007; Neel et al., 2003). By contrast, the inhibitory actions of HGF on satellite cell proliferation as mediated through SHP2 are independent of sustained ERK1/2 phosphorylation. High concentrations of HGF cause an initial rise in phosphoERK1/2 that is absent within 20 min post-stimulus. The downstream SHP2 targets in satellite cells remain unknown but may include p190RhoGAP. SHP2
associates with p190RhoGAP and dephosphorylation of the GTPase activating protein allows for the activation of RhoA in C2C12 myoblasts (Kontaridis et al., 2004). RhoA can phosphorylate and activate phosphotidylinositol 3-kinase (PI3K) and downstream Akt to promote myoblast survival (Reuveny et al., 2004). In addition, constitutive expression of RhoA in C2 myoblasts impedes both MyoD expression and muscle fiber formation. HGF/MET activation of RhoA is integral to epithelial cell migration and invasion (Kitajo et al., 2003; Miao et al., 2003). Future pursuits may include an examination of HGF's ability to initiate Rho-dependent satellite cell proliferation and growth arrest.

Coincident with a role in cell cycle kinetics, SHP2 also is integral to stem cell biology and self-renewal. In trophoblasts, SHP2 protects the putative stem cell from apoptosis during early embryogenesis (Yang et al., 2006). Mouse SHP2<sup>−/−</sup> blastocysts contain a trophectoderm layer that fails to implant into the uterine wall and lacks differentiated giant cells. Importantly, trophectoderm-derived stem cell lines cannot be established in vitro in the absence of a functional SHP2. In a similar manner, targeted deletion of SHP2 in neural progenitor cells compromises FGF2-mediated self-renewal through repression of Bmi-1 expression, a polycomb protein implicit in self-renewal of multiple stem cell lineages (Ke et al., 2007; Pardal et al., 2005). By contrast, others report that FGF signaling through FRS2a and SHP2 is critical for neural stem and progenitor cell proliferation in vitro but dispensable for self-renewal (Yamamoto et al., 2005). The absolute requirement of a functional SHP2 for continuous expansion of stem cells is further complicated by reports that deletion of the phosphatase in ES cells results in improved rates of secondary embryoid body formation, a measure of self-
renewal (Qu and Feng, 1998). SHP2 null ES aggregates exhibited reduced expression of MyoD and myogenin suggesting that fewer skeletal myoblasts were formed and differentiated by comparison to wild-type. It is unknown if a Pax3, Pax3:Myf5 or Pax7 expressing myogenic population forms in SHP2\(^{(-/-)}\) embryoid bodies. Each of these populations can yield a MyoD-expressing myoblast (Bajard et al., 2006; Relaix et al., 2005). Treatment of satellite cells with HGF, which signals through downstream SHP2, tended to increase the numbers of Pax7 immunopositive cells and reduce the percentage of cells expressing MyoD. Satellite cells defined as Pax7\(^{+}\) and Myf5/MyoD\(^{-}\) are capable of asymmetric self-renewal in vitro and repopulation of the niche following transplantation into the tibialis anterior muscle (Kuang et al., 2007). A shift toward a population enriched for Pax7\(^{+}/\)MyoD\(^{-}\) cells suggests that SHP2 is important for satellite cell self-renewal. Conditional ablation of SHP2 in MyoD and/or Pax7 expressing cells will assist in defining the role of the phosphatase during adult myogenesis.

The involvement of SHP2 during myofiber formation is context dependent with the phosphatase participating in both promotional and inhibitory signals (Kontaridis et al., 2002; Koyama et al., 2008). SHP2 activity increases during the transition from mononucleated myoblast to multinucleated myofiber (Kontaridis et al., 2004). However, ectopic expression of constitutive active SHP2 represses C2C12 muscle fiber formation (Kontaridis et al., 2002). Ablation of SHP2 in satellite cells in vitro delays myogenesis resulting in small neofibers with fewer than 5 myonuclei thus, supporting a role for the phosphatase during myoblast fusion (Fornaro et al., 2006). Our results indicate a temporal role of SHP2 for induction of biochemical and phenotypic differentiation. Given that SHP2 activity increases during fiber formation, our results are suggestive that
sustained activity accelerates 23A2-SHP2-CA muscle fiber maturation as denoted by a reduction in myogenin expression with no effect on morphological parameters. The correct timing of SHP2 activity may underlie the divergent responses of immortal and primary myoblasts. In data not shown, relative amounts of SHP2 are greater in primary satellite cells than in 23A2 myoblasts. Primary satellite cells are notorious for spontaneous differentiation even in the presence of high fetal bovine serum concentrations (10-15%). In this scenario, the elevated SHP2 may predispose the cells to accelerated myogenesis under conditions that normal suppress C2C12 and 23A2 myogenesis. The reasons for divergent behavior of myoblasts to a common growth factor or intracellular signaling intermediate are unknown. However, we provide evidence that conventional myoblast lines are not duplicitous of satellite cells and caution against direct extrapolation of results. During the course of spontaneous immortalization, myoblasts may have acquired a refractile nature to signals that would disrupt cell cycle kinetics or invoke quiescence in a primary myogenic cell. A phosphoproteomic assessment of HGF-induced signaling intermediates that underlie opposing phenotypic responses in the two myogenic cell types will provide insight into both the effects of signal intensity and cell immortalization.

In summary, we provide evidence that HGF participates in satellite cell G0 entry through a mechanism that involves SHP2. Knockdown of SHP2 improves proliferation rates and ectopic SHP2 inhibits satellite cell cycle kinetics. Importantly, SHP2 causes divergent effects on cell proliferation that is dependent upon the myogenic background. Manipulation of SHP2 activity may allow for the creation of myogenic cells that are more suited for engraftment into compromised skeletal muscle.
Figure 3-1. All SHP2 myoblasts cell lines express abundant amounts of fusion proteins. 23A2 myoblasts were stably transfected with plasmids coding for wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA), or dominant negative SHP2 (SHP2-DN). Total cellular protein lysates were examined by Western blot for FLAG-tagged SHP2 expression.
Figure 3-2. SHP2 stimulates proliferation in myoblasts. 23A2 myoblasts were stably transfected with plasmids coding for wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or dominant negative SHP2 (SHP2-DN). Subconfluent cultures of these myoblasts were pulsed for 30 minutes with BrdU to measure the numbers of cells traversing S-phase. Myoblasts expressing SHP2-WT and SHP2-CA contain a higher percentage of proliferating cells (*; P<0.05).
Figure 3-3. Activated SHP2 inhibits myofiber formation. 23A2 myoblasts were stably transfected with plasmids coding for wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or dominant negative (SHP2-DN). These myogenic cells were induced to differentiate for 48 hours and immunostained for myosin heavy chain. All cell types form multinucleated myofibers but the myofibers formed from 23A2-SHP2-CA are smaller.
Figure 3-4. Activated SHP2 inhibits myogenin expression. 23A2 myoblasts were stably transfected with plasmids coding for wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or dominant negative (SHP2-DN). These myogenic cells were induced to differentiate for 48 hours and lysed for Western blot-scanning densitometry analysis for myogenin expression. Comparing with other cells, 23A2-SHP2-CA had a lower myogenin expression (*, P<0.05).
Figure 3-5. Hepatocyte growth factor (HGF) induced proliferation requires SHP2. Control and 23A2 myoblasts expressing wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or dominant negative (SHP2-DN) were treated with HGF (10 ng/ml) for 48 hours prior to measurement of the numbers of BrdU immunopositive cells. HGF induces proliferation in control cells that is blocked by SHP2-DN (*, P<0.05). Means and SEMs of a minimum of three independent experiments are shown.
Figure 3-6. Hepatocyte growth factor (HGF) stimulates ERK1/2 activation independent of SHP2. 23A2 myoblasts expressing wild-type SHP2 (SHP2-WT), constitutive active SHP2 (SHP2-CA) or dominant negative (SHP2-DN) were treated for 48 hours with HGF (10 ng/ml). The ability of HGF to stimulate ERK1/2 was examined in the respective cell lines by Western blot (A). A transient peak of phosphoERK1/2 is present within 10 minutes. The relative intensities of the phosphoERK1/2 bands were normalized to tubulin content and plotted (B). Neither SHP2-WT nor SHP2-CA extends the phosphoERK1/2 signal.
Figure 3-7. Hepatocyte growth factor (HGF) and SHP2 stimulate proliferation independent of ERK1/2. The myogenic cell lines were treated for 48 hours with HGF (10 ng/ml) in the presence or absence of 25 μM PD98059. Cells were pulsed with BrdU (30 minutes) and the numbers of BrdU immunopositive cells were measured. Means and SEMs of a minimum of three independent experiments are shown.
Figure 3-8. SHP2 does not affect satellite cell proliferation and activation. Satellite cells were transiently transfected with CMV or CMV-SHP2-WT and a GFP-expression plasmid. After 48 hours, the cells were immunostained for the proliferation marker, Ki67. The numbers of GFP-expressing cells that contain Ki67 were enumerated.
Figure 3-9. Mouse satellite cells (MSCs) expresses a higher concentration of phospho-SHP2 than 23A2 myoblasts. MSCs and 23A2 myoblasts were lysed and evaluated by Western blot for SHP2 and phosphor-SHP2 expression. MSCs expressed equivalent amount of SHP2 protein as 23A2 myoblasts. However, the active forms of SHP2 were reduced in 23A2 myoblasts.
Figure 3-10. SHP2 protein expression was knockdown by SHP2 siRNA. pSirenRetroQ-siCon and pSirenRetroQ–siSHP2 were transfected into 23A2 myoblasts and evaluated by Western blot for SHP2 content after 48 hours. SHP2 siRNA causes a reduction in SHP2 protein expression.
Figure 3-11. Blockage of SHP2 promotes satellite cell proliferation. pSirenRetroQ-siCon and pSirenRetroQ–siSHP2 were transfected into mouse satellite cells. After 48 hours, the GFP expressing cells were examined for Ki67 expression. A reduction in SHP2 protein causes an increase in proliferation of satellite cells (*, P<0.05).
Figure 3-12. High concentrations of HGF induced arrest of mouse satellite cells. Satellite cells were treated with 10 or 50 ng/ml HGF for 48 hours prior to measurement of proliferation. The percentage of cells incorporating BrdU is shown. The lower concentration of HGF (10 ng/ml) stimulated cell proliferation. The higher concentration of HGF (50 ng/ml) caused a significant reduction in the numbers of BrdU-positive cells (*, P<0.05).
Figure 3-13. HGF induced arrest is mediated by SHP2. Satellite cells were transiently transfected with pSirenRetroQ-siCon or –siSHP2 and treated for 48 hours with 50 ng/ml HGF. The percentage of GFP expressing cells containing Ki67 was measured. The inhibitory effect of HGF is overcome by removal of SHP2.
Figure 3-14. HGF induces ERK1/2 activation independent of phosphor-SHP2. Satellite cells were treated with 50 ng/ml HGF for up to 30 minutes. Expression of ERK1/2, phosphor-ERK1/2, SHP2, and phosphor-SHP2 were examined by Western blot. A transient peak of phosphoERK1/2 is present within 10 minutes, and disappears after 20 minutes. Satellite cells express a constant amount of Phospho-SHP2, which is not affected by HGF treatment.
Figure 3-15. HGF induced arrest requires ERK1/2 activation. Satellite cells were treated with or without 50 ng/ml HGF in the presence or absence of 25 μM PD98059 for 48 hours prior to measurement of proliferation. The percentage of cells incorporating BrdU is shown. Either blocking ERK1/2 with inhibitor or HGF treatment alone caused a decreased proliferation rate (*, P<0.05).
CHAPTER 4
EVIDENCE OF HETEROGENEITY WITHIN BOVINE SATELLITE CELLS ISOLATED FROM YOUNG AND ADULT ANIMALS

Satellite cells are a group of cells located between sarcolemma and basal lamina of the host fiber (MAURO, 1961), which have the potential of proliferation, differentiation and self-renewal (Beauchamp et al., 1999). Both in human and rodent models, satellite cells exhibit an age-associated decline in cell number, as well as myogenic capability (Conboy et al., 2003; Schmalbruch and Hellhammer, 1976; Snow, 1977).

Evidence from mouse model demonstrates that mouse satellite cells (MSCs) are not a homogeneous pool; instead, they exist as a heterogeneous population exhibiting distinct biological functions (Collins et al., 2005; Kuang et al., 2007). During muscle regeneration, portions of satellite cells become activated and express some myogenic regulatory factors, such as Myf5 and MyoD. These committed muscle progenitors undergo several rounds of proliferation before terminal differentiation (Charge and Rudnicki, 2004). However, a subpopulation of muscle stem cells is always maintained through self-renewal (Kuang et al., 2008; Montarras et al., 2005).

Heterogeneity has been well studied in MSCs via different methods (Beauchamp et al., 2000; Fukada et al., 2004; Hashimoto et al., 2004; Kuang et al., 2007; Montarras et al., 2005; Zammit et al., 2002). Pax7, a lineage marker of satellite cells, is required for maintenance of skeletal muscle in embryonic and fetal development (Lepper et al., 2009; Seale et al., 2000). Myf5-Cre lineage tracing demonstrates that 10% of Pax7-positive MSCs have never expressed Myf5 and act as a stem cell subpopulation, whereas the other 90% of Pax7: Myf5 co-expressing cells are the committed progenitors (Kuang et al., 2007). Pax7-expressing satellite cells divide in asymmetric manner to give rise to a Pax7+/Myf5+ muscle progenitor daughter, as well as a
Pax7+/Myf5− muscle stem cell daughter for self-renewal (Kuang et al., 2007). Although Pax7 marks majority of satellite cells, a combination of other markers, such as Pax3, Myf5, CD34 and M-cadherin, are also used to define heterogeneity of satellite cells (Beauchamp et al., 2000; Montarras et al., 2005; Relaix et al., 2005; Zammit et al., 2002). In addition, different sub-groups of satellite cells are also accessed in other animal species, such as avian and human (Pawlikowski et al., 2009; Rouger et al., 2004). However, heterogeneity of satellite cell in meat producing animals, such as cattle, is still unclear.

Bovine satellite cells (BSCs) activate, proliferate, and differentiate similarly to other species, such as rodent and human (Allen et al., 1991). BSCs express lineage marker Pax7 (Gonzalez et al., 2007), as well as the myogenic regulatory factors (Muroya et al., 2005). However, BSCs isolated from postnatal animal do not express desmin, a traditional marker for mononucleated rat satellite cells, which suggests BSCs may exhibit subtle differences from rodents (Allen et al., 1991).

The objective of the study was to identify the level of heterogeneity within BSCs. BSCs exhibit a similar myogenesis process as MSCs, and the myogenesis progression shows an age-associated delay. Further analysis of Pax7 and Myf5 expression indicates BSCs constitute of Pax7+/Myf5+ muscle progenitor cells, Pax7+/Myf5− muscle stem cells, and Pax7−/Myf5+ myoblasts. Moreover, clonal analysis demonstrates that BSCs segregate into fast-growing and slow-growing colonies. Fast-growing colonies are derived from the committed progenitors, which co-express Pax7 and Myf5, whereas slow-growing colonies are derived from stem-like cells. Both symmetric and asymmetric divisions of Pax7-only stem cells are noted from clonal result. Interestingly, a group of
slow-growing and Myf5-only stem cell is identified, and it shows neither Pax7 expression nor intrinsic myogenic potential. In summary, these studies get an assessment of the heterogeneity of BSCs, and provide evidence for the complexity of stem cell population within BSCs.

**Materials and Methods**

**Bovine Satellite Cell Isolation**

Young Holstein bull calves (< 7 days of age) or adult Holstein heifers (>30 months of age) were used for satellite cell isolation. Animals were euthanized following the procedure approved by University of Florida Institutional Animal Care and Use Committee. Following captive bolt stun and exsanguinations, the semimembranosus (SM) and longissimus dorsi (LD) muscle were removed, with connective tissue and adipose tissue dissected, and then ground with meat processor. Ground muscle tissue was digested with 1 mg/ml protease (Sigma Aldrich) for 45 min at 37°C, with agitation every 10 min. The slurry was centrifuged at 1500 x g for 4 min to remove protease. The pellets were resuspended in PBS, vortexed, and centrifuged at 500 x g to collect satellite cells in the supernatant. The PBS wash was repeated 3 times and the supernatants were centrifuged at 1500 x g for 10 min. Satellite cell pellets were reconstituted in growth medium and sequentially filtered through 70um and 40um cell strainer (Invitrogen). The filtered cells were stored in liquid nitrogen with 10% DMSO for later use.

**Cell Culture**

Primary bovine satellite cells (BSCs) were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% horse serum (Invitrogen)
1% penicillin-streptomycin (Invitrogen), and 0.5% gentamycin on entactin-collagen-laminin (ECL, Invitrogen) coated tissue culture dishes.

Clones were established by serial dilution to achieve a single cell in each well of a 96-well, ECL-coated culture plate. Population doubling times were calculated using the formula:

\[ N(t) = C(2)^{t/d} \]
\[ d = 10/\log(N) \]

\( N(t) \) = the number of cells at time \( t \); \( d \) = doubling time; \( c \) = initial number of objects; \( t \) = time

**Immunocytochemistry**

BSCs were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized and nonspecific binding sites were blocked with 5% horse serum in PBS containing 0.1% Triton X-100. Cells were further incubated overnight at 4 °C in rabbit anti-Myf5 (1:50, Santa Cruz), mouse anti-MyoD (1:30, Vector Laboratories), mouse anti-Pax7 (1:10 hybridoma supernatant, Developmental Studies Hybridoma Bank), mouse anti-myogenin (1:1000 hybridoma supernatant, Developmental Studies Hybridoma Bank), or mouse anti-myosin heavy chain (MF20, 1:5 hybridoma supernatant, Developmental Studies Hybridoma Bank). After exhaustive wash with PBS, the cells were incubated with goat anti-mouse AlexaFluorv488 (1:250, Invitrogen) or/and goat anti-rabbit AlexaFluor 527 (1:150, Invitrogen) for 1h at room temperature. Total nuclei were detected with Hoechst 33342 (Invitrogen). Immune complexes were visualized with Nikon TE2000 inverted phase microscope equipped with epifluorescence. Representative images were captured with a CoolPix CCD camera and analyzed with NIS-Elements software (Nikon).

Proliferation of BSCs was measured through Click-iT Edu cell proliferation kit (Invitrogen). Cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton
X-100 after pulse with 20 uM Edu for 2 h. Subsequently, cells were incubated with reaction cocktail containing Alexa Fluor azide 527 for 30 min.

Statistics

All data were analyzed by one-way ANOVA followed by t-test using Statistical Analysis System (SAS Institute Inc., Cary, NC). Results are presented as the mean ± SEM. A p-value<0.05 was considered to be significant.

Results

BSCs Exhibit an Age-dependent Decline in Mitogenesis and Myogenesis.

In rodent models, satellite cells down-regulate Pax7 and up-regulate MyoD during activation, as well as up-regulate myogenin as they differentiate (Halevy et al., 2004; Zammit et al., 2004). Rat satellite cells also exhibit an age-dependent decline in mitosis in vitro (Conboy et al., 2003). In this study, we cultured BSCs from young (<7 day) and adult (>24 month) cattle. The cell number (Figure 4-1), DNA synthesis (Figure 4-2), MyoD (Figure 4-3) and myogenin (Figure 4-4) expression were measured daily. BSCs from young animals started DNA synthesis after 48 hours of culture, and proliferated after 72 hours of culture. MyoD, a marker of activation, was expressed about 72 hour after culture, and differentiation marker myogenin was expressed after 120 hours of culture. Similar to rodent satellite cells, there is an age-related decline in the activation period, which is denoted by increased Edu incorporation and cell number, as well as in the myogenesis period, which is marked by MyoD and myogenin expression. With time in culture, BSCs fuse to form multinucleated myofibers (Figure 4-16).

BSCs are a Heterogeneous Population

In mice, Pax7⁺/Myf5⁻ cells represent quiescent muscle stem cells, while Pax7⁺/Myf5⁺ cells represent committed muscle progenitors responsible for myogenesis
Three distinct subpopulations of BSCs were identified by co-immunostaining of Pax7 and Myf5 (Figure 4-5). The majority of BSCs co-expressed Pax7 and Myf5 within the nucleus, suggesting their commitment to myogenic lineage. These cells were maintained for 3 days of culture, whereas the cell percentage dropped at day 4, when differentiation markers started to appear (Figure 4-7). The second population of cells only expressed Pax7, indicating that they represent the muscle stem cells found in mice (Figure 4-5, green arrow). The Pax7-only stem cells accounted for 5% of total BSCs throughout the culturing time (Figure 4-6). Interestingly, a small number of BSCs only expressed Myf5 (Figure 4-5, red arrow). The number of these Myf5-only myoblasts began to increase at day 4, in parallel with myogenic differentiation and Pax7/Myf5 double-positive cell decline (Figure 4-8).

Satellite cells from different muscles also have different marker expression indicating heterogeneity (Montarras et al., 2005; Relaix et al., 2005; Relaix et al., 2006). To identify different subpopulations of BSCs in different muscle, BSCs were isolated from both semimembranosus (SM) and longissimus dorsi (LD) muscle of young calves. Co-expression of Pax7 and Myf5 in SM and LD muscle revealed the similar subpopulations. However, LD muscle contained a lower percentage of muscle progenitors and a higher percentage of myoblasts (Figure 4-9).

**Extensive Degree of Heterogeneity Exists in Slow-growing Muscle Stem Cells.**

To further define BSC heterogeneity, clonal analysis was performed on BSCs isolated from SM of young animals (n=4). Single cell clones were established in individual wells of 96-well plates and cultured for 10 days. Total numbers of cells per colony (well) were used to calculate population-doubling time (PDT). The results showed that BSCs exhibited different proliferation efficiency, with PDT ranging from 1
day to more than 10 days (Figure 4-10). Although nearly 90% of the colonies were regarded as fast-growing colonies with PDT less than 3 days, 10% of colonies were slow-growing with PDT more than 3 days (Figure 4-11). The fast-growing clones differentiate and fuse to large myofibers after 10 days of culture, suggesting their strong myogenic potential. The slow-growing BSC clones offered additional features, such as small and light refractile morphology, which was distinct from their fast-growing counterparts. It has been reported that similar types of muscle stem cells exist in other species (Hashimoto et al., 2004; Montarras et al., 2005). Thus, there is a possibility that the fast-growing colonies are derived from Pax7/Myf5 double positive muscle progenitor cells that contribute to muscle growth, whereas the slow-growing colonies are derived from Pax7-only muscle stem cells that maintain the stem cell pool.

To further evaluate the stem-like clones, slow-growing clones were immunostained for expression of Pax7 and Myf5. Initial scoring of the clones revealed that the slow-growing cells were a mix population constituting of variety of cell types, including Pax7-only, My5-only, Pax7/Myf5 positive-positive and Pax7:/Myf5 double-negative colonies (Figure 4-12). No significant differences in the relative abundance of subtypes exist between animals. Results demonstrate that an extensive degree of heterogeneity with regard to lineage marker expression exists within the slow-growing stem cells. Pax7-only stem cells in mouse exhibit both symmetric and asymmetric division, contributing to either myogenesis or self-renewal (Kuang et al., 2007). It is found that Pax7-only stem cells performed both division manners, similar to mouse satellite cells (Figure 4-13, 4-14). Of the 53 colonies expressing Pax7 and Myf5, 7 were derived from asymmetric division, with the remaining colonies comprised of Pax7/Myf5 double-positive cells from
symmetric division. Interestingly, approximately 15% of the clones contained two or more Pax7-only cells, indicating the existence of planar division of Pax7-only stem cells.

Among all the slow-growing clones of stem cells, the most intriguing subclass is the Myf5-only cells. These cells represented nearly one-half of all slow-growing clones (Figure 4-12), and had a higher PDT than Pax7-expressing clones (Figure 4-15). In addition, different from other subpopulations, Myf5-only cells proliferated but failed to differentiate and fuse to multinucleated myofibers (Figure 4-16, white arrow).

Discussion

Animal Model for Satellite Cell Research

BSCs are similar to cultured rat SCs. Both exhibit a time-course of myogenic lineage progression, and an age-related delay of activation and differentiation (Grounds, 1998; Johnson and Allen, 1995). In addition, rodent satellite cells, with or without their associated fibers, exhibit MyoD expression prior to DNA synthesis (Beauchamp et al., 2000; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2004). However, BSCs isolated from young cattle start to enter S-phase at day 2, whereas they do not express MyoD until day3. These results indicate that BSCs express MyoD later during proliferation, a subtle difference from rodent satellite cells.

In mice, Pax7 and Myf5 expression defines two distinct subpopulations of satellite cells, Pax7\(^+\)/Myf5\(^-\) stem cells and Pax7\(^+\)/Myf5\(^+\) muscle progenitors (Kuang et al., 2007). By contrast, my results showed BSCs were composed of three subgroups of cells based on expression of Pax7 and Myf5. Pax7/Myf5 double-positive cells and Pax7-only cells represent the corresponding subgroups in MSCs. However, immunostaining results in this research showed 5% of BSC only express Myf5, indicating that there is a subgroup of myoblasts within BSC. Since the Myf5-only subpopulation is present in both
young and adult, it is unlikely that they are remnant fetal myoblasts. However, the origin and function of these cells is still unclear.

**Heterogeneity of Bovine Satellite Cell**

It has been reported that at least two subgroups of satellite cells exist in other species, such as rat, chicken and turkey (Rouger et al., 2004; Schultz, 1996; Yablonka-Reuveni et al., 1987). Comparing the results from immunostaining with clonal analysis in this study, the fast-growing clones exhibited some similar characteristics as Pax7/Myf5 double-positive cells, such as protein expression and proliferation rate. These results indicate that fast-growing clones are derived from the Pax7/Myf5-positive cells, which represent the muscle progenitors in other species (Kuang et al., 2007; Yablonka-Reuveni et al., 1987). In addition, the fast-growing clones showed strong intrinsic differentiation capability, consistent with the myogenesis progression in Pax7/Myf5 double-positive cells. By contrast, a portion of the slow-growing clones expressed Pax7, and they also exhibited a small, round, and light refractile shape, similar to Pax7-only cells from immunostaining. These characteristics are also reported to exist in muscle stem cells from other species (Hashimoto et al., 2004). As a result, the Pax7-expressing, slow-growing clones may derive from the Pax7-only stem cells. Moreover, both symmetric and asymmetric divisions were denoted in the Pax7-expression clones in my study, indicating the presence of self-renewal that is also seen in mouse satellite cells (Kuang et al., 2007). However, immunostaining results showed a sustained percentage of Pax7-only stem cells, suggesting that the number of stem cell increases at the same rate as proliferation. Comparing to stem cells in mass culture system, a single stem cell, without interaction with other cells, chooses self-renewal over myogenesis. Differences in growth rate of stem cells between mass culture system
and single-cell clonal system provide a potential mechanism, which can be used for further analysis of fate-determination factors from cell-cell interaction.

**Asymmetric Division Independent of Satellite Cell Niche**

Satellite cell self-renewal through symmetric division usually depends on the satellite cell niche. Kuang et al. shows during asymmetric division, one satellite cell close to the basal lamina becomes Pax7-only stem cell, and the other cell dividing apically, away from the basal lamina, becomes Pax7/Myf5 duo-positive progenitor cell (Kuang et al., 2007). In addition, some niche associated factors, such as Notch-1 and its antagonist Numb, are also related to DNA segregation and fate determination of satellite cells (Conboy et al., 2003; Shinin et al., 2006). Thus, the satellite cell niche, including the basal lamina, plays an important role in regulating asymmetric division and fate determination of satellite cells. However, our clonal analysis provides additional evidence for asymmetric division of satellite cells without their associated fibers. A very small percentage of colonies with both Pax7-only cells and Pax7/Myf5 duo-positive cells exist. These colonies may arise from a single Pax7-only stem cell that divides asymmetrically. The similar result is also reported in mice and human satellite cells (Pawlikowski et al., 2009; Shinin et al., 2006). These results suggest satellite cells are able to divide asymmetric and adopt different cell fate independent of the associated fibers.

**Characteristics of Myf5-only Myoblast**

The most intriguing subgroup of BSCs found in this research was defined by Myf5 expression only. These cells constituted over one-half of all slow-dividing clones, and were morphologically indistinguishable from Pax7-expressing muscle stem cells. The
morphology and protein expression suggests these Myf5-only clones may derive from the initial 5% Myf5-only cells in mass culture system.

However, it has been reported that Myf5 expression is regulated by Pax3, a paralog of Pax7, which is required for migration of myoblast into the developing limb area (Bajard et al., 2006; Goulding et al., 1994; Relaix et al., 2005). Due to the lack of reliable antibody, expression of Pax3 is unable to be traced in these cells. It has been reported that mouse hindlimb muscles contain few Pax3 expressing cells by comparison to forelimb muscle and postural muscles of the back (Montarras et al., 2005). My result indicates a more abundant population of Myf5-only cells, comparing to the Pax3/Myf5 population in mouse satellite cells, suggesting a distinct populations present in cattle but absent in mouse. Previous experiment in human satellite cells (HSCs) indicates that 5% of HSCs are negative for Pax7 but positive for N-CAM, another reliable marker for HSCs (Lindstrom and Thornell, 2009). The NCAM+/Pax7 subgroup of HSCs may be similar to the Myf5-only cell in BSCs. As a result, Myf5-only subpopulation of BSCs may represent a similar population of myoblasts in human, which is absent in mouse.

The genetic fingerprint of these cells remains elusive, it is possible that they are derived from a Pax7 precursor or alternatively, the cells may have expressed the lineage marker prior to isolation. Pax7 mRNA contains target regions for miRNA-1 and miRNA-206 that regulate satellite cell proliferation and differentiation by repressing Pax7 expression (Chen et al., 2010). There is a possible mechanism that repression of Pax7 expression in Myf5-only BSCs caused by miRNA is responsible for their fate determination. However, the origin and function of the Myf5 BSC are still under investigation.
Similarity of Bovine Satellite Cells and Human Satellite Cells

It is reported in human satellite cells (HSCs), 95% of HSCs are positive for both Pax7 and N-CAM, 1% of HSCs are positive for Pax7 but negative for N-CAM, and 5% of HSCs are negative for Pax7 but positive for N-CAM (Lindstrom and Thornell, 2009). Since N-CAM labels the regenerating muscle progenitors in human (Schubert et al., 1989), which is similar to Myf5 in rodent and bovine, the Pax7+/N-CAM− and Pax7+/N-CAM+ subpopulations in HSCs may represent the corresponding Pax7+/Myf5− and Pax7+/Myf5+ cells we find in BSCs. The NCAM+/Pax7− subgroup of HSCs may be similar to the Myf5-only cell in BSCs. Thus, the myoblast subpopulation of satellite cells may exist in human and bovine, which is absent in mouse.

Furthermore, Pawlikowski et al. find a group of differentiation resistant satellite cells in human (Pawlikowski et al., 2009). The differentiation resistant cells have a low percentage of BrdU incorporation after 10 days of culture (6%). Half of these differentiation resistance cells express Pax7, and the other half do not. When these differentiation resistance cells are induced for the second round of differentiation, large myofibers are formed. It seems the differentiation resistant cells represent the stem cells with myogenic potential. These stem cells share similar characteristics as our slow-glowing stem cells, including 50% Pax7-negative population, low mitogenesis and resistant to first round differentiation. In addition, less than 1% of these stem cells maintain Pax7 expression following second round of differentiation compared to 8% with the first round. It suggests a possibility that Pax7-positive stem cells tend to differentiate at second round, and the Pax7-negative cells, similar to our Myf5-only clones, are resistant to second round of differentiation. Thus, considering the similarity of stem cell
heterogeneity and characteristics, bovine satellite cells may be a better animal model to analyze the cellular and molecular mechanism in human satellite cells.

In summary, BSC are a heterogeneous population that fall into three distinct subgroups based upon expression of Pax7 and Myf5. The Pax7-only and Pax7/Myf5 double-positive populations appear similar to the rodent muscle stem and progenitor cells, respectively. Unlike rodent satellite cells, a unique population of satellite cells that express Myf5 is identified. This group of cells is retained into adulthood and may represent a Pax3-expressing satellite cell. The heterogeneous model of bovine satellite cells is summarized in Figure 4-17. These results offer insight into the diversity of the satellite cell population in cattle and provide a framework for future efforts defining the optimal cell type for postnatal muscle growth.
Figure 4-1. Bovine satellite cells exhibit an age-dependent reduction in cell growth. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 5 days. The cell number was calculated. Satellite cells from adult animal exhibited lower cell number after 72 hours of culture (*, \( P<0.05 \)). Means and SEMs of 3 independent experiments are shown.
Figure 4-2. Bovine satellite cells exhibit an age-dependent decline in proliferation. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 5 days. The cells were fixed daily and analyzed for Edu incorporation. Satellite cells from adult animal exhibited a decreased Edu incorporation rate at 48 or 72 hours of culture (*, P<0.05). Means and SEMs of 3 independent experiments are shown.
Figure 4-3. Bovine satellite cells display an age-dependent decline in MyoD expression. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 5 days. The cells were fixed and analyzed for MyoD expression. Satellite cells from adult animal exhibited a decreased MyoD expression after 72 hours of culture (*, P<0.05). Means and SEMs of 3 independent experiments are shown.
Figure 4-4. Bovine satellite cells display an age-dependent decline in myogenin expression. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 6 days. The cells were fixed and analyzed for myogenin expression. Satellite cells from adult animal exhibited a decreased myogenin expression after 120 hours of culture (*, P<0.05). Means and SEMs of 3 independent experiments are shown.
Figure 4-5. Bovine satellite cells are composed of three sub-populations based on Pax7 and Myf5 expression. BSCs isolated from semimembranosus muscle of young (<7 days) animals were cultured in ECL-coated plate for 48 hours prior to fix and stain for Pax7 and Myf5 expression. Red arrow indicates cells express Myf5 only, and green arrow represents cells express Pax7 only.
Figure 4-6. Bovine satellite cells from young and adult animals have similar percentage of Pax7-only cells. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 6 days prior to fix and stain for Pax7 and Myf5 expression. Percentage of Pax7-only cells was calculated. There is no difference in percentage of Pax7-only cells. Means and SEMs of 3 independent experiments are shown.
Figure 4-7. Bovine satellite cells from young animals exhibit a faster decline of Pax7:Myf5 dual-positive cell percentage. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 5 days prior to fix and stain for Pax7 and Myf5 expression. Percentage of Pax7:Myf5 dual-positive cells was calculated. Means and SEMs of 3 independent experiments are shown.
Figure 4-8. Bovine satellite cells from young animals exhibit a higher percentage Myf5-only cell. BSCs isolated from semimembranosus muscle of young (<7 days) and adult (>30 months) animals were cultured in ECL-coated plate for 5 days prior to fix and stain for Pax7 and Myf5 expression. Percentage of Myf5-only cells was calculated. Means and SEMs of 3 independent experiments are shown.
Figure 4-9. BSCs heterogeneity varies in different muscles. BSCs isolated from semimembranosus (SM) and longissimus dorsi (LD) muscle of young (<7 days) animals were cultured in ECL-coated plate. Cells were cultured for 48 hours before fixed and stained for Pax7 and Myf5 expression. Percentage of 3 subpopulations was calculated. Means and SEMs of 3 independent experiments are shown.
Figure 4-10. Bovine satellite cells have different growth rates. BSCs isolated from SM muscle of young animals were culture in ECL-coated 96-well plates at single cell density. After 10 days of culture, population doubling time (PDT) of each colony was calculated. Means and SEMs of 3 animals are shown.
Figure 4-11. Bovine satellite cells contain two sub-types based on different growth rates. BSCs isolated from SM muscle of young animals were culture in ECL-coated 96-well plates at single cell density. After 10 days of culture, population doubling time (PDT) of each colony was calculated. Colonies were segregated into two categories: slow-growing colony (PDT>3) and fast-growing colony (PDT<3). Means and SEMs of 3 animals are shown.
Figure 4-12. Extensive degree of heterogeneity exists among slow-growing colonies. Previous described slow-growing colonies were fixed and stained for Pax7 and Myf5 expression. Initial scoring of Pax7-only, Myf5-only, Pax7:Myf5 duo-positive and Pax7:Myf5 null clones revealed the presence of different subtypes within slow-growing cells.
Figure 4-13. Pax7 expression muscle stem cell divided through both symmetric and asymmetric manner. Previous described slow-growing colonies with two cells were analyzed for Pax7 and Myf5 expression. Immuno-staining result indicated Pax7-only cells divided through both symmetric and asymmetric manners.
Figure 4-14. Pax7 expression muscle stem cell divided through both symmetric and asymmetric manner. Previous described slow-growing colonies with two cells were analyzed for Pax7 and Myf5 expression. Immuno-staining result indicated Pax7-only cells divided through both symmetric and asymmetric manners. Pax7 positive colonies were segregated based on their division pattern.
Figure 4-15. Myf5-only cells proliferate faster than Pax7-only cells. Slow-growing colonies contain both Myf5-only colonies and Pax7 colonies. After 10 days of culture, population doubling time (PDT) of each colony was calculated. Pax7-only cells have higher PDT than Myf5-only cells.
Figure 4-16. Myf5-only cells do not differentiate or fuse to myofibers. BSCs were cultured in ECL-coated plate for 5 days until they began to differentiate and fuse to myofibers. Cells were fixed and stained for Myf5 and myosin heavy chain. White arrow showed that Myf5 expressing cells did not express myosin heavy chain or fuse to myofibers.
Figure 4-17. Bovine satellite cells are heterogeneous populations. Bovine satellite cell is composed of three subpopulations: Pax7\(^+\)/Myf5\(^-\) (A), Pax7\(^-\)/Myf5\(^+\) (B), and Pax7\(^+\)/Myf5\(^+\) (C). Pax7\(^+\)/Myf5\(^+\) cell is the muscle progenitor, which proliferates fast and finally differentiates to form myonuclei. Pax7\(^+\)/Myf5\(^-\) cell is a type of muscle stem cell, which either divides through symmetric manner to give rise to two Pax7\(^+\)/Myf5\(^+\) progenitors for myogenesis, or divides through asymmetric manner to give rise to one Pax7\(^+\)/Myf5\(^+\) progenitor, and one Pax7\(^-\)/Myf5\(^-\) stem cell for self-renewal. Pax7\(^+\)/Myf5\(^-\) cell is a special type of muscle stem cell in bovine, which does proliferate but does not differentiate. The special function of Pax7\(^-\)/Myf5\(^+\) cell is still unclear.
CHAPTER 5
EPHRIN-A5 REGULATES PRIMARY BOVINE SATELLITE CELLS MIGRATION THROUGH RAC1/RHOA SIGNALING

During embryonic development, the ephrin-Eph system plays an essential role in muscle patterning and innervation (Kilpatrick et al., 1996; Ohta et al., 1996). Ephrin ligands A2 and A5 along with their receptor EphA3, EphA4, and EphA5 are expressed in the spinal cord and the limb during delamination and migration of myogenic precursor cells (Iwamasa et al., 1999). The relationship between EphA4 expression and limb muscle mass suggests the ephrin system directs migration of muscle precursors (Iwamasa et al., 1999). Ephrin-A2, EphA4, and EphA7 expression in skeletal muscle persists from late fetal stage to adulthood (Lai et al., 1999; Lai et al., 2001). Interestingly, their expression tends to concentrate in the postsynaptic apparatus from postnatal day 7 to day 21, coincident with neuromuscular junction (NMJ) functional remodeling (Bewick et al., 1996). EphA4 and EphA7 in adult muscle are regulated through a nerve-dependent manner (Lai et al., 2001). These results indicate that the ephrin system plays a role in adult muscle function.

Ephrin-A5 regulates the migration and attachment of neural cells (Frisen et al., 1999; Wilkinson, 2001), endothelial (Stein et al., 1998), and neuroendothelial cells (Holmberg et al., 2000). During skeletal muscle development, ephrin-A5/EphA4 interaction limits the migration of Pax7-positive muscle precursor cells from lateral dermomyotome to limb buds (Swartz et al., 2001). Since the transcription pattern and protein localization are different between embryonic and adult muscle (Iwamasa et al., 1999; Lai et al., 2001), the action of ephrin-A5 on adult satellite cells may be different from embryonic precursors. Thus, the function of ephrin-A5 on satellite cell migration is still unclear.
Ephrin-A5 causes auto-phosphorylation of EphA receptor tyrosine kinases (RTKs), which in turn activate downstream effector, Rho GTPases (Wahl et al., 2000). Rho GTPases play an essential role in ephrin-A5-induced regulation of actin cytoskeleton rearrangement, which causes neural growth cone collapse and neural retraction (Kranenburg et al., 1999). In this research, the function of ephrin A-5 and its receptors in the migration of primary BSCs was studied. Both dimeric and multimeric ephrin-A5 act as attractive cues for primary BSCs but not for myoblast. Lineage tracing experiments suggest that ephrin-A5 only regulates a portion of satellite cells prior to activation. Further analysis also indicates Rho family GTPases work downstream of ephrin-A5, which is required for ephrin-A5-induced chemoattractive effects. Moreover, these results provide a possible molecular mechanism of satellite cell maintenance in adult muscle, as well as denervation-induced muscle atrophy.

**Materials and Methods**

**Animal Care and Satellite Cells Isolation**

All animals were handled according to approved practices from the University of Florida Institutional Animal Care and Use Committee. Holstein bull calves less than 7 days of age were used for satellite cell isolation. Following captive bolt stun and exsanguination, the hindlimb semimembranosus muscles were removed, with connective tissue and adipose tissue dissected. Then muscle was cut to small pieces and ground with commercial meat processor. Ground muscle tissues were digested with 1 mg/ml protease (Sigma Aldrich) for 45 min at 37 °C, with agitation every 10 minutes. The slurry was centrifuged at 1500 x g for 4 minutes to remove the protease. The pellets were resuspended in PBS, vortexed and centrifuged at 500 x g for 10 minutes. The PBS wash was repeated 3 times and supernatants were collected each time. The
supernatants were centrifuged 1500 x g for 10 minutes, and satellite cell pellet was resuspended in growth medium before filtered sequentially through 70 μm and 40μm cell strainer (Invitrogen). Cells were collected by centrifugation and resuspended in growth medium supplemented with 10% DMSO, and stored in liquid nitrogen until needed.

**Cell Culture**

23A2 myoblasts were cultured on gelatin-coated tissue culture plates in Basal medium Eagle (BME) containing 15% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin, and 0.2% gentamicin. Bovine satellite cells were cultured on ECL-coated tissue culture dishes in low glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum (HS) and antibiotics. All cell culture medium and supplements were purchased from Invitrogen. Recombinant Human Ephrin-A5/Fc (R&D Systems) was clustered with anti-human IgG (Jackson Immuno-research) or AlexaFluor 488 anti-human IgG (Invitrogen) for 20min at 37°C. Human Fc fragment (Jackson Immuno-Research) was used as a negative control. After clustering, ephrin-A5 was supplied at 5 μg/ml in growth medium. HGF was supplied to cells at 10 ng/ml. Inhibition of MEK1/2, Rac1 and RhoA activities was accomplished by supplementation of culture medium with 50 μM PD98059 (Cell Signaling), 50 μM Rac1 inhibitor (Calbiochem) and 50 μM Y-27632 (Calbiochem).

**Western Blot**

All cells were lysed in 4X sample buffer (250mM Tris, pH 6.8, 8% SDS, 40% glycerol, and 0.4% β-mercaptoethanol), briefly sonicated, and heated at 95 °C for 5 minutes. Proteins were separated through 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with 5% non-fat dried milk in TBST
(10mM Tris, pH8.0, 150mM NaCl, and 0.1% Tween-20) for 30 min to block non-specific binding sites. Then blots were incubated overnight with anti-ERK1/2, anti-phosphoERK1/2, anti-Akt, anti-phosphoAkt (1:1000, Cell Signaling), anti-EphA2, anti-EphA4 (1:100, Santa Cruz) or for 1 hour at room temperature with anti-tubulin (1:5000, Abcam). After extensive washes with TBST, the blots were incubated with appropriate peroxidase-conjugated secondary antibody for 1 hour, following by chemiluminescent detection through ECL kit (Amersham Biosciences) and exposure to X-ray film.

**Immunocytochemistry**

All cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature. Non-specific antigen sites were blocked with PBS containing 5% horse serum and 0.1% Triton X-100. Cultures were incubated with anti-EphA2, EphA4, anti-Myf5 (1:50, Santa Cruz), anti-MyoD (1:30, Vector Laboratory), anti-Pax7 (1:10 Hybridoma Bank), or anti-MyHC (1:20, Hybridoma Bank) at room temperature for 1 hour. After exhaustive rinses with PBS, the fixed cultures were incubated goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 527 (Invitrogen) for 1 hour. Cultures were counterstained with Hoechst 33342 (Invitrogen) for the visualization of nuclei and phalloidin (Invitrogen) for actin filament. Immunofluorescence was detected with Nikon TE 2000 inverted phase microscope equipped with epifluorescence. Represent pictures were captured with CoolPix CCD Photometrics digital camera, and analysis with NIS-Element software.

**Time-lapse Photography**

Bovine satellite cells were cultured at ECL-coated glass bottom cell culture dishes in growth medium. After cells attached to the surface, the dishes were transferred to incubation chamber (37 °C and 5% CO2) linked to LiveCell™ System (Pathology
Devices Inc, MD). The time-lapse images were captured with Nikon TE 2000 inverted phase microscope and Cool SNAP Photometrics digital camera. The images were analysis with NIS-Element software and NIH Image J software.

**Transwell Migration Assay**

ECL-coated transwell inserts (24-well, pore size 8 μm, Corning Inc., MA) were seeded with 3000 cells in 200 μl heat-inactive growth medium. Heat-inactive growth medium with or without clustered ephrin-A5 or HGF was added to the lower chamber and served as a chemotactic agent. After 4 or 24 hours of culture, non-migrating cells were wiped from the upper side of the membrane and cells on the lower side were fixed with 4% paraformaldehyde for 15 minutes. Nuclei were stained with Hoechst 33342 (Invitrogen) and counted as migrating cell number. Each individual treatment was repeated three times with duplicate inserts and 8 microscopic fields were counted per insert.

**Statistics**

All data were analyzed by one-way ANOVA followed by t-test using Statistical Analysis System (SAS Institute Inc., Cary, NC). Results were presented as the mean ± SEM. A p-value<0.05 was considered to be significant.

**Results**

**Bovine Satellite Cells Exhibit Variant Pattern of Motility in vitro.**

Mouse satellite cells move on surfaces coated with ECM proteins, such as laminin, fibronectin, collagen and elactin (Echtermeyer et al., 1996; Ocalan et al., 1988; Siegel et al., 2009; Yao et al., 1996). Time-lapse videography reveals that BSCs are able to move on the elactin-collagen-laminin (ECL)-coated surface. Cultured BSCs exhibit different motion patterns that are correlated with their sizes (Figure 5-1, Figure 5-3). The
large, wide-spread cells tend to move faster than the small, round transparent cells. In order to test if different subpopulations of BSCs have different motility during in vitro culture, BSCs were fixed and co-stained with Pax7 and Myf5 antibodies after the time-lapse image capture. The results showed BSCs were also composed of Pax7+/Myf5+ committed progenitors and Pax7+/Myf5- stem cells. When tracing the motility of different sub-populations of cells, the result indicated that stem cells moved slower compared to committed progenitors (Figure 5-2). These results were consistent with the very low cytoplasm to nuclei ratio and transcriptional level of quiescent muscle stem cells (Biressi and Rando, 2010; Kuang et al., 2008). BSC stem cells moved less than 2 μm per hour with occasional stretches, which suggested they were alive but with limited proteins expression (Figure 5-3). Thus, BSCs exhibit different motility related their lineage.

**Ephrin-A5 is an Attractive Cue for BSCs Migration**

Ephrin-A5 and its receptors are expressed in both embryonic and adult skeletal muscle (Iwamasa et al., 1999; Lai et al., 2001). It regulates migration of avian myogenic precursors during limb development (Swartz et al., 2001). Western blot result showed EphA2 and EphA4, receptors of ephrin-A5, were expressed in both primary BSCs and 23A2 myoblasts (Figure 5-4). When fluorescence-linked IgG was used to precluster ephrin-A5/Fc, multimeric ephrin-A5 complexes were formed and able to be traced via fluorescence microscopy. The result indicates ephrin-A5 binds to it receptors on cell surface (Figure 5-5).

Ephrin-A5 has been reported as both attractive and repellent cues in various cell types (Frisen et al., 1999; Holmberg et al., 2000; Stein et al., 1998; Swartz et al., 2001; Wilkinson, 2001). When Ephrin-A5/Fc was supplemented to the growth medium, it dimerized, evenly distributed over the cell surface, and limited BSCs motility during 4-
hour treatment. Random motility of 23A2 myoblasts is unaffected (Figure 5-6). To further define ephrin-A5 as an attractive or repulsive cue for BSC migration, transwell migration assays were performed. Since dimeric and multimeric ephrins have distinct effects on epithelial cell migration and adhesion (Stein et al., 1998), either dimerized ephrin-A5/Fc (Figure 5-8) or multimerized preclustered ephrin (Figure 5-7) were tested for their capability to induce cell migration. Both forms of ephrin-A5 significantly increased the number of BSCs that migrated through the membrane towards the ligands. The increased migration cell percentage reveals the chemoattractive function of ephrin-A5 during BSC migration, which is opposite to its chemorepellent function during embryonic development of limb muscle (Swartz et al., 2001). There was no difference between the dimeric and multimeric forms of ephrin in regulation of BSC migration. Neither form of ephrin-A5 exhibited an effect on 23A2 myoblast migration.

**Only Muscle Progenitors are Affected Prior to Activation.**

Ephrin-A5 directs the migration of 20-30% of BSCs, suggesting that only a subpopulation of cells is responsive. The velocities of committed progenitors and stem cells were separated based on their marker expression. The results showed that only the motility of Pax7+/Myf5+ cells was regulated by ephrin-A5; whereas the motility of Pax7+/Myf5- muscle stem cells was unaffected (Figure 5-9). Thus, ephrin-A5 only limits the motility of committed muscle progenitors.

The effect of ephrin-A5 on BSC motility at different stages of myogenic progression was examined. BSCs were cultured for 24 or 96 hours, non-enzymatically removed from the plates and seeded atop transwell filters. Immunostaining results indicated BSCs cultured for 24 hours did not express any activation marker, MyoD; whereas more than 60% BSCs cultured for 96 hours were activated with MyoD.
expression (Figure 5-11). When these cells were applied for transmigration analysis, data showed 96-hour-cultured BSCs had a limited migration capability. They also did not respond to ephrin-A5 in contrast to 24-hour-cultured BSCs (Figure 5-10). Thus, ephrin-A5 only affects BSCs prior to their activation. In summary, ephrin-A5 only attracts committed progenitors before they go through myogenesis.

**Ephrin-A5 Regulates BSCs Migration Independent of ERK1/2 Signaling.**

Ephrin-A5 acts as a chemoattractive cue for BSCs but not for 23A2 myoblasts. The different responses between them may be due to different intracellular pathways. EphA RTKs interact with a series of downstream proteins, including phosphotyrosine-binding adaptors (Grb2, Gab1, crk, Nck), PDZ domain proteins, PI3K subunit, and modulators of Ras and Rho family small GTPases (Lai and Ip, 2003). Raf-MEK-ERK1/2 signaling and PI3K-Akt signaling are major pathways downstream of general RTKs. These two pathways are required for satellite cell migration induced by various factors (Ratajczak et al., 2003). In 23A2 myoblasts, ephrin-A5 induced a transient activation of ERK1/2 at 5 minutes with a secondary peak of phosphorylated ERK1/2 at 20 minutes (Figure 5-12). However, ephrin-A5 did not activate PI3K pathway through phosphorylating Akt. By contrast, ephrin-A5 could not activate either ERK1/2 or Akt in BSCs (Figure 5-13). Considering the previous results indicating that ephrin-A5 only promotes migration of BSCs but not 23A2, ephrin-A5’s regulation of BSC migration may require blockage of transient activation of ERK1/2. To test this hypothesis, ephrin-A5 induced trans-migration of BSCs and 23A2 was examined in the presence or absence of ERK1/2 activation (Figure 5-14). The result indicated inhibition of ERK1/2 activation did not affect the migration of BSCs (Figure 5-15); whereas inhibition of ERK1/2 activity tended to increase migration of 23A2 myoblasts independent of ephrin-A5 stimulation.
Thus, ephrin-A5 stimulates BSCs migration independent of ERK1/2 activation. Moreover, ERK1/2 signaling may be involved in myoblast migration, although it is not related to Ephrin-A5 stimulation.

HGF is a chemotaxis factor for mouse satellite cells, which regulates satellite cell migration during muscle injury (Bischoff, 1997; Bladt et al., 1995; Corti et al., 2001; Hill et al., 2006; Lee et al., 1999). HGF induced ERK1/2 activation in BSCs (Figure 5-16). Further analysis of HGF regulation of BSC migration showed HGF decreased BSCs motility after 24-hour treatment (Figure 5-17), as well as served as a chemoattractant for BSCs migration (Figure 5-18). These results demonstrate HGF and ephrin-A5 regulates the migration of BSCs in a similar way, although these factors exhibit completely different pattern of ERK1/2 activation.

**Ephrin-A5 Regulates BSCs Migration through Rac1/RhoA Signaling.**

Rho family of small GTPases, including three members Rac1, Cdc42 and RhoA, are important regulators of actin cytoskeleton arrangement downstream of Eph RTKs (Noren and Pasquale, 2004; Schmitz et al., 2000). Activation of Rho GTPases and its downstream effector Rho kinase is required for ephrin-A5-induced collapse of growth cones (Wahl et al., 2000). Moreover, although Rac1 and RhoA are usually involved in different cellular processes, they can act in an opposite or competitive manner when targeting the same cellular response (Kozma et al., 1997). EphA regulates axon guidance by modulating the balance between Rac1, Cdc42 and RhoA signaling, with RhoA and Rac1 acting in an opposite way during this process (Shamah et al., 2001). To identify the importance of Rho GTPases during ephrin-A5-induced migration of BSCs, the trans-migration of BSCs in response to ephrin-A5 was analyzed with and without Rac1 or RhoA inhibitors. Both Rac1 and RhoA GTPase are suggested to be required for
ephrin-A5 induced migration (Figure 5-19). Thus, Rac1 and RhoA share some common functions downstream of ephrin-A5-induced migration of BSCs.

Internalization, achieved by rapid assembly and disassembly of actin filaments, is essential to remove Eph-ephrin complex from cell surface and enables the dissociation of cells (Egea and Klein, 2007). Rho GTPases regulate cell morphology and motility through assembly and arrangement of the actin cytoskeleton (Aspenstrom et al., 2004). To trace ephrin-A5 internalization, Ephrin-A5/Fc was clustered with fluorescence-linked IgG prior to incubation with BSCs and 23A2 myoblasts. Time-lapse images showed ephrin-A5 complex rapidly entered the cytoplasm of 23A2 myoblasts. The fluorescent signal disappeared after 4 hours, suggesting inactivation or degradation of ephrin-A5-receptor complexes. By comparison, ephrin-A5 exhibited a delayed internalization in BSCs (Figure 5-20). Quantitive analysis of fluorescence intensity contained within the cell showed that BSCs and 23A2 myoblasts had different patterns of ligand internalization (Figure 5-21). However, inhibition of Rac1 or RhoA signals did not change the internalization pattern of BSCs (Figure 5-22), indicating that receptor internalization is not directly related to Rho GTPase-mediated migration of BSCs.

**Discussion**

During skeletal muscle development, components of ephrin-Eph RTK signaling are expressed in the embryonic limb muscle (Flenniken et al., 1996; Iwamasa et al., 1999; Klein, 2004; Lai et al., 2001). They play an essential role in muscle patterning and innervation, as well as neuromuscular junction remodeling during embryonic development (Eberhart et al., 2000; Iwamasa et al., 1999; Kilpatrick et al., 1996; Lai et al., 2001; Ohta et al., 1996). Furthermore, ephrin-A5 acts as a repulsive cue for Pax7-positive muscle precursors in vitro and in vivo, demonstrating that ephrin-A5 guides
EphA4-positive muscle precursor cells to migrate towards the appropriate territories in forelimb (Swartz et al., 2001). In this study, EphA2 and EphA4 were abundantly expressed in bovine satellite cells, suggesting they have additional functions in adult muscle. Moreover, ephrin-A5 accumulated on the cell membrane, and subsequently entered cytoplasm. This observation suggests a potential for attractive effect of ephrin-A5.

Both dimeric and multimeric ephrin-B1 activate EphB1 receptor. However, only multimeric ephrin-B1 promotes the attachment of endothelial, whereas dimeric form of ephrin-B1 shows no effect (Stein et al., 1998). When the function of dimeric ephrin-A5/Fc and multimeric forms of ephrin was compared in this research, there was no difference in the regulation of BSCs migration, or activation of ERK1/2 and Akt substrates. As a result, dimeric and multimeric ephrin-A5 have no difference in regulation of BSCs migration, suggesting downstream signal of ephrin-A5 depends on the EphA receptors. Thus, to further analyze ephrin-A5 signaling, the intracellular signal induced by EphA2 and EphA4 should be separated.

Ephrin ligands, including ephrin-A5, are well known for their repulsive effects on neural cells and EphA4-expressing muscle precursor cells (Egea and Klein, 2007; Swartz et al., 2001). Surprisingly, ephrin-A5 acted as an attractive cue for BSCs in this study. Their attractive or adhesive effects have been reported in several other systems (Davy et al., 1999; Hansen et al., 2004; Holmberg et al., 2000; Pandey et al., 1995; Stein et al., 1998). Cranial neural cells expressing both EphA7 and ephrin-A5 adhere to each other and adhesion is impaired in the absence of either EphA7 or ephrin-A5 (Holmberg et al., 2000). One possible mechanism for the dual-directional effect of
ephrin on cell migration is based on the receptor expression in target cells. Ephrin-A5 interacts with EphA7 in cranial neural cells. EphA7 encodes one full-length receptor and two truncated receptors lacking the kinase domain in the neural cells. Full-length EphA7 are repelled by clustered ephrin, whereas truncated EphA7 causes cell adhesion to ephrin-A5 predominantly (Holmberg et al., 2000). Consistent with the previous idea, ephrin-Eph RTKs signal depends on the Eph receptor instead of the ligands.

Ephrin-A5-induced cell migration is mediated by rearrangement of actin cytoskeleton (Huang et al., 1997; Kranenburg et al., 1999) via Rho family GTPases (Huang et al., 1997; Wahl et al., 2000). Three members of Rho family GTPases, RhoA, Rac1, and Cdc42, regulate actin cytoskeleton dynamics through different manners. For example, RhoA regulates stress fiber and focal adhesion formation and cell contractility, whereas Rac1 and Cdc42 activation results in the formation of protrusive structures such as lamellipodia and filopodia respectively (Noren and Pasquale, 2004). Ephrin-A5 signaling induces collapse of neuronal growth cones through up-regulation of RhoA and down-regulation of Rac1, suggesting that Rac1 and RhoA play different roles in regulation of cytoskeleton organization (Shamah et al., 2001; Wahl et al., 2000). However, some GTPase modulators, such as Ras-GAP (Ras GTPase-activating proteins) and Ephexin (a Guanine nucleotide exchange factor, GEF), are involved in ephrin-dependent corporation of Rho family GTPase activities (Holland et al., 1997; Shamah et al., 2001). Ephrin-A5 induced migration of BSCs was blocked by either Rac1 or RhoA inhibition in my study. However, Rac1 or RhoA treatment without ephrin-A5 stimulation did not affect migration of BSCs. These results suggest both Rac1 and RhoA signals are required for ephrin-A5-induced migration, and they regulate cell
motility through association with activated EphA RTKs via GTPase modulators. Moreover, it is a possible that Ephrin-A5 regulates Rac1 and RhoA through different manners via distinct GTPase regulators, which cause the similar downstream actions on cell migration.

Results of this study show neither RhoA nor Rac1 effects ephrin-A5-receptor internalization. One possible explanation is that BSCs express very low amount of actin filaments and keep minimal rate of actin recycling, which is consistent with the results that random motility of BSCs is low, and EphA2/4 has a positive staining in the cytoplasm. The minimal actin production and recycling is required for BSCs survival, which can not be limited by Rho GTPase family inhibitors.

Ephrin-A5, EphA4 and EphA2 are expressed in the neuromuscular junction of adult muscle (Lai et al., 2001). EphA4 expression and NMJ functional remodeling share the similar time-course (Bewick et al., 1996). Moreover, cortactin, a downstream component of EphA4, modulates cytoskeleton reorganization in postsynaptic apparatus (Huang et al., 1997) and AchR clusters in NMJ (Dai et al., 2000). As a result, ephin-A5 may play a role in NMJ formation and muscle innervation. In this study, the ephrin-A5-directed migration of committed progenitors was found in BSCs. This result demonstrates a possible mechanism that ephrin-A5 preserves the committed progenitors within NMJ as an emergency cell pool to keep them from activation. When surrounding myofibers are injured, elimination of ephrin-A5 from the NMJ causes rapid release of the muscle progenitors, which migrate from the donor muscle fiber to the injury site in response to factors, such as HGF and NO. Moreover, long-term denervation with NMJ degradation causes released satellite cells to migrate towards the
interstitial space, leading to loss of muscle mass (Borisov et al., 2005). However, the function of NMJ in satellite cell activity requires further investigation.

In summary, BSCs are able to move on the coated surface in vitro. The motility of BSCs is regulated by ephrin-A5 in a Rac1/RhoA-dependent manner. Moreover, ephrin-A5 acts as a chemo-attractive cue for committed progenitors prior to activation.
Figure 5-1. Bovine satellite cells have variable motility. BSCs were cultured in ECL-coated plates for 24 or 48 hours before captured for a 4 hours time-lapse movie. Average velocities were measured with NIH Image J software, and cell sizes were measured with NIS-Element software. Each spot indicated one BSC with corresponding velocity and size. Motility of BSCs is positively related to cell size.
Figure 5-2. Bovine satellite cells have variable motility. BSCs were cultured in ECL-coated plates for 24 or 48 hours before captured for a 4 hours time-lapse movie. Average velocities were measured with NIH Image J software. At the end of the time-lapse movie, the BSCs were fixed and stained for Pax7 and Myf5. Velocities of cells expressing different markers were measured. Pax7+/Myf5+ cells move faster than Pax7+/Myf5- cells (p<0.05). Velocity of Pax7+/Myf5+ cells increases with culture time (*, p<0.05). Means and SEMs of 3 or more independent experiments are shown.
Figure 5-3. Slow-moving BSCs are living cells that capable of stretching. BSCs were cultured in ECL-coated plates for 24 or 48 hours before captured a 4 hours time-lapse movie. White arrow indicates the same cell at different times.
Figure 5-4. EphA2 and EphA4 are expressed in 23A2 myoblasts and bovine satellite cells (BSCs). 23A2 myoblasts and BSCs were cultured in growth medium for 24h in ECL-coated plates. EphA2 and EphA4 expression and localization are examined by Western blot. Both 23A2 myoblasts and BSCs express abundant of EphA2 and EphA4 proteins.
Figure 5-5. EphrinA5 binds to its receptor on the cell membrane. Bovine satellite cells (BSCs) were cultured in growth medium for 24h in ECL-coated plates. Ephrin-A5 was clustered with AlexFluor 488 before supplemented to the BSCs for 10 minutes. Ephrin-A5-AlexFluor 488 complex is located at the cell membrane.
Figure 5-6. Ephrin-A5 changes motility of bovine satellite cells (BSCs). 23A2 myoblasts and BSCs were cultured in ECL-coated plates for 24 hours before replacement of growth medium with or without 5ug/mL of ephrin-A5. Four-hours time-lapse movies were captured after treatment. Average velocities were calculated with NIH Image J software. Ephrin-A5 decreases the motility of BSCs, but not the 23A2 (*, p<0.05). SEMs of at least 3 independent experiments are shown.
Figure 5-7. Multimeric Ephrin-A5 acts as a chemo-attractant for bovine satellite cells (BSCs). 23A2 and BSCs were seeded in ECL-coated transwell insert, and heat-inactive growth medium with or without pre-clustered ephrin-A5 (5ug/mL) were added to the lower well. Numbers of cells migrate through the membrane were calculated after 4 hours incubation. Ephrin-A5 increases the percentage of migrating BSCs, but it does not affect 23A2 migration (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-8. Dimeric Ephrin-A5 acts as a chemo-attractant for BSCs. 23A2 and BSCs were seeded in ECL-coated transwell insert, and heat-inactive growth medium with or without non-clustered ephrin-A5 (5ug/mL) were added to the lower well. Numbers of cells migrate through the membrane were calculated after 4 hours incubation. Ephrin-A5 increases the percentage of migrating BSCs, but it does not affect 23A2 migration (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-9. Ephrin-A5 only affects muscle progenitors. BSCs were cultured in ECL-coated plates for 24 hours before replacement of growth medium with or without 5 ug/ml of preclustered ephrin-A5/Fc. Four-hour time-lapse movies were captured before the cells were fixed and stained for Pax7 and Myf5 expression. Average velocities were calculated with NIH Image J software. Means and SEMs of at least 3 independent experiments are shown.
Figure 5-10. Ephrin-A5 only affects BSCs prior to long-term culture. BSCs were cultured in ECL-coated plates for 24 or 96 hours prior to scraped and resuspended in heat-inactive growth medium. The cells were either seeded in ECL-coated transwell insert, with heat-inactive growth medium with or without preclustered ephrin-A5 (5μg/mL) added to the lower well. Numbers of cells migrate through the membrane were calculated after four-hours incubation. Ephrin-A5 increases migration rate of cells cultured for 24 hours, but not cells cultured for 96 hours (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-11. Bovine satellite cells express MyoD after long-term of culture. BSCs were cultured in ECL-coated plates for 24 or 96 hours prior to scraped and resuspended in heat-inactive growth medium. The cells were either seeded in ECL-coated transwell insert, with heat-inactive growth medium with or without preclustered ephrin-A5 (5ug/mL) added to the lower well. After 4 hours of culture, Migrating cells were fixed and attained for MyoD. After 96 hours of culture, 60% of cells expressed MyoD, and the percentage of MyoD-positive cells is not affected by ephrin-A5 (p>0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-12. Ephrin-A5 activates ERK1/2 in 23A2 myoblasts. 23A2 myoblasts were cultured in growth medium for 24 hours prior to serum starvation and then treated with 5μg/mL preclustered ephrin-A5. Total cell lysates of 23A2 myoblasts were evaluated by Western blot for ERK1/2 and Akt activity. Ephrin-A5 induces a transient ERK1/2 phosphorylation at 5 minutes, but it does not cause Akt phosphorylation.
Figure 5-13. Ephrin-A5 does not activate ERK1/2 or Akt in bovine satellite cells (BSCs). BSCs were cultured in growth medium for 24 hours prior to serum starvation and then treated with 5μg/mL preclustered ephrin-A5. Total cell lysates were evaluated by Western blot for ERK1/2 and Akt activity. Ephrin-A5 does not cause phosphorylation of either ERK1/2 or Akt.
Figure 5-14. PD98059 causes blockage of ERK1/2 activation. Bovine satellite cells (BSCs) and 23A2 myoblasts were cultured for 24 hour before treated with or without 50uM PD98059 for 4 hours. Total cell lysate were analyzed by western blot for total and phosphor-ERK1/2 expression. PD98059 blocks ERK1/2 activation in both 23A2 myoblasts and BSCs.
Figure 5-15. Blockage of ERK1/2 activation does not affect ephrin-A5-induced migration of bovine satellite cells (BSCs). BSCs and 23A2 were cultured for 24 hour before treated with or without 50uM PD98059 for 4 hours. Then these BSCs and 23A2 were seeded in ECL-coated transwell insert, with heat-inactive growth medium with or without preclustered ephrin-A5 (5ug/mL) added to the lower well. Numbers of cells migrate through the membrane were calculated after 4 hours incubation. Ephrin-A5 induced cell migration in BSCs does not affected by PD98059 (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-16. HGF activates ERK1/2 activation. BSCs were cultured in growth medium for 24 hours prior to serum starvation and then treated with 10ng/mL HGF. Total cell lysates were evaluated by Western blot for ERK1/2. HGF activates ERK1/2 transiently at 5 minutes, and then the phosphor-ERK1/2 decreases after 10 minutes of treatment.
Figure 5-17. HGF changes motility of BSCs after 24 hours of treatment. BSCs were treated with growth medium presence or absence of 10 ng/mL of HGF. Four-hour time-lapse movies were captured 4h or 24h after treatment. Average velocities were calculated with NIH Image J software. HGF causes a decline of cell velocity after 24 hours of treatment (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-18. HGF promotes migration of bovine satellite cells (BSCs). BSCs were seeded in ECL-coated transwell insert, and heat-inactive growth medium with or without 10ng/ml HGF were added to the lower well. Numbers of cells migrate through the membrane were calculated after 4 hours or 24h of incubation. HGF increases the migration of BSCs after 24 hours of treatment (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-19. Ephrin-A5 promotes BSCs migration through Rac1/RhoA signaling. BSCs were treated with heat-inactive growth medium presence or absence of Rac1 inhibitor or RhoA inhibitors, and then seeded in transwell insert with heat-inactive growth medium with or without preclustered Ephrin-A5 (5ug/ml) in the bottom well. Numbers of cells migrate through the membrane were calculated after four-hour incubation. Ephin-A5 induced cell migration is blocked by Rac1 or RhoA inhibitors (*, p<0.05). Means and SEMs of at least 3 independent experiments are shown.
Figure 5-20. Ephrin-A5 is internalized in bovine satellite cells (BSCs) and 23A2 myoblasts. 23A2 and BSCs were cultured for 24 hours prior to treatment with Ephrin-A5 clustered with AlexFluor 488. Cells were fixed at 5 min, 10 min, 30 min, 60 min, and 4 h after treatment. Images were captured with cell membrane and nuclei outlined. Ephrin-A5 is internalized in both cell types, but it sustains in BSCs for a longer period.
Figure 5-21. Ephrin-A5 internalization is delayed in bovine satellite cells (BSCs) compared to 23A2 myoblasts. 23A2 and BSCs were cultured for 24 hours prior to treatment with Ephrin-A5 clustered with AlexFluor 488. Cells were fixed at 5min, 10min, 30min, 60min, and 4h after treatment. Images were captured with cell membrane and nuclei outlined. Fluorescence signal density (pix/um2) was measured with NIS-Element software. Fluorescence signal density in 23A2 drops down after 4 hours. However, fluorescence density in BSCs sustained after 4 hours. Means and SEMs at least 40 cells from 3 independent experiments are shown.
Figure 5-22. Rac1/RhoA signaling does not affect Ephrin-A5 internalization in bovine satellite cells (BSCs). BSCs were cultured for 24 hours before treated with Ephrin-A5 clustered with AlexFluor 488 in the present or absent of Rac1 or RhoA inhibitors. Cells were fixed at 4 hours after treatment, and fluorescence density was measured. Fluorescence density does not affected by Rac1 or RhoA inhibitors (p>0.05). Means and SEMs of at least 3 independent experiments are shown.
Skeletal muscle represents over 40% of the body’s mass and is responsible for posture and movement. The number of myofibers is fixed at birth, and postnatal muscle growth is a result of muscle hypertrophy. Muscle hypertrophy is caused by increasing sizes of the individual myofibers through addition of myonuclei and increased protein synthesis. Satellite cells, the muscle-restricted stem cell, are the major sources of myonuclei for postnatal muscle growth. In newborn animals, satellite cells comprise approximately 30% of all muscle nuclei within a given muscle. Their numbers decline to approximately 2-6% at adulthood (Cardasis and Cooper, 1975; Snow, 1977). The age-related decline in satellite cell numbers is associated with a decrease in mitotic activation, proliferation, differentiation, and migration (Charifi et al., 2003).

In the adult animal, satellite cells are mitotically quiescent and need to be activated prior to cell cycle reentry. After several rounds of proliferation, a small portion of the satellite cells withdraws from cell cycle and return to the quiescent state. Many growth factors and steroids are used in the beef industry that may improve satellite cell activity. For example, implants containing trenbolone acetate (TBA) and estradiol-17β (E₂) cause an increase in carcass weight, which maybe related to increased satellite cell activity (Chung and Johnson, 2008; Johnson et al., 1998). IGF-I and FGF2 are the best known factors that are able to stimulate satellite cell proliferation, while members of TGF-β family like myostatin are responsible for negative regulation of satellite cells activity (Allen and Rankin, 1990; Johnson and Allen, 1990). HGF is another important regulator of satellite cells, since it is the only growth factor that stimulates satellite cell activation as well as subsequent proliferation (Miller et al., 2000; Sheehan et al., 2000).
In addition, HGF is reported to regulate satellite cell differentiation (Gal-Levi et al., 1998; Leshem et al., 2000; Miller et al., 2000) and migration (Bischoff, 1997; Corti et al., 2001; Hill et al., 2006; Yanagiuchi et al., 2009). In addition, HGF is involved in regulation of satellite cell activity after exercise or muscle injury (Tatsumi et al., 2001; Tatsumi et al., 2002; Tatsumi et al., 2006).

C-met is the receptor for HGF (Rosario and Birchmeier, 2003). However, multiple intracellular signals downstream of c-met are initiated and serve to modify cellular functions. In this study, we find that SHP2 is an important mediator in primary mouse satellite cells, which responds to different concentration of HGF and initiates the proliferation program. Our results are consistent with a recent study demonstrating that high concentration of HGF inhibits satellite cell proliferation by inducing myostatin expression (Yamada et al., 2010). Myostatin is the major limitation factor for muscle growth. Mutation in myostatin gene causes double muscling phenotype in both mice and cattle (McPherron et al., 1997; McPherron and Lee, 1997). As a result, this study provides a glance of the mechanism for growth limitation in meat producing animals. Intracellular molecules like SHP2 may exist as modulators that switch between distinct intracellular signals in response to circulating or domestic hormone levels.

Since satellite cells play an essential role in postnatal growth of skeletal muscle, a model for satellite cell study in meat producing animal is required. Satellite cell heterogeneity is well understood in rodent model (Kuang et al., 2007; Montarras et al., 2005), and accessed in human satellite cells (Lindstrom and Thornell, 2009). However, no previous research shows distinct subpopulations of satellite cells in cattle or other large animals. In this study, an assessment of the heterogeneity in bovine satellite cells
was obtained. BSCs are composed of sub-groups of cells with different marker expression pattern, growth rate, and motility. Not only the mass cultured BSCs are heterogeneous, but also the stem cell subgroup within BSCs has distinct marker expression. The myoblast stem cell population may exist in BSCs, which grows faster compared to typical muscle stem cells, but may not be capable of differentiation and contributing to muscle growth. The function of these redundant cells in the skeletal muscle of cattle remains a mystery. However, the extra stem cell pool within skeletal muscle brings a potential to unlock the growth limitation for cattle.

Considering the similarities in anatomy and physiology, bovine satellite cells may be a better model for human satellite cell therapies than rodent. Limited migration capability of transplanted satellite cell challenges regeneration efficiency (Skuk et al., 1999). Systemic factors involved in regulation of satellite cell migration, such as HGF, bFGF, IGF-1, PDGF and IL-4, are well studied, (Bischoff, 1997; Corti et al., 2001; Hill et al., 2006; Horsley et al., 2003; Lafreniere et al., 2006; Robertson et al., 1993; Yanagiuchi et al., 2009). However, some factors within the satellite cell niche possibly affect satellite cell localization and migration as well. Ephrin-A5-EphA2/4 system within neuromuscular junction may play an important role in maintenance of satellite cells in adult muscle. Muscle injury-induced migration of satellite cells is known to require ephrin-A5 system. In addition, the ephrin-A5 system may be involved in muscle denervation and satellite cell dysfunction associated with neuromuscular diseases or aging.
APPENDIX A
SATELLITE CELL ISOLATION PROTOCOL

Adult Balb-C female mice are used for mouse satellite cell isolation.

1. Muscle groups from the upper hindlimb and back are harvested from adult mice and placed in sterile PBS.
2. Fat and connective tissues are removed from the excised muscles.
3. The muscle tissues are finely minced with scissors in 10 cm plastic dish, and digested with 2X volume of protease type XIV solution (1.5 mg/ml protease in PBS).
4. The protease-muscle solution is incubated in 37 °C water bath for 60 minutes, with vortexing every 15 minutes.
5. The proteinase is removed by centrifugation of the tissue slurry at 1500 x G for 5 minutes.
6. An equal volume of warm PBS is added to the tissue, vortexed for 5 minutes and centrifuged at 400 x G for 5 minutes.
7. The process is repeated for a total of three times with the supernatants retained after each centrifugation.
8. The supernatants are centrifuged at 1500 x G for 5 minutes to collect the cells, and then solved in growth medium (high-glucose DMEM, 10% HS, 1% penn/strep, 0.2% gentamycin).
9. The cells are passaged sequentially through 70 μm and 40 μm filters to remove debris, and then collected by centrifugation.
10. The isolated satellite cells are either seeded in ECL-coated tissue cultureware or stored frozen in liquid nitrogen.
Holstein bull calves less than 7 days or more than 24 month of age are used for bovine satellite cell isolation.

1. Hindlimb semimembranosus muscles are removed by sterile knife, and placed in cold PBS.

2. The connective tissue and adipose tissue are trimmed off with scissors, and the muscle was cut to small pieces and ground with commercial meat processor.

3. Ground muscle tissues are digested with equal volume of 1 mg/ml protease XIV in 37°C water bath (45 minutes for young animals and 60 minutes for adult animals, with agitation every 10 minutes).

4. The slurry is centrifuged at 1500 x G for 4 minutes in room temperature to remove the protease.

5. The pellets are resuspended in warm PBS, vortexed and centrifuged at 500 x G for 10 minutes.

6. The PBS wash is repeated 3 times and supernatants were collected each time. The supernatants are centrifuged 1500 x G for 10 minutes, and satellite cell pellet is resuspended in growth medium (low-glucose DMEM, 10% HS, 1% penn/strep, 0.2% gentamycin).

7. The Slurry is filtered sequentially through 70 μm and 40 μm cell strainer.

8. Cells were collected by centrifugation at 1500 x G for 10 minutes, and resuspended in growth medium.

9. The isolated satellite cells were either seeded in ECL-coated tissue cultureware or stored frozen in liquid nitrogen.
APPENDIX B
SDS-PAGE AND WESTERN BLOT PROTOCOL

Samples Preparation

1. Cells growing in culture dishes are washed twice with ice cold Tris buffered saline (TBS) [with 1mM phenylmethanesulfonylfluoride (PMSF)].

2. Add SDS-PAGE sample buffer with 1mM protease inhibitor (PMSF, NaF, Na4P2O7, Na3VO4), scrape and transfer into microcentrifuge tube.

3. Heat at 95 °C for 5 minutes, and sonicate for 30 seconds.

4. Centrifuge at 12000 rpm for 30 seconds, and aliquot the supernatant for SDS-PAGE.

SDS-PAGE and Transfer

1. Samples are separated through polyacrylamide gels. The recipe for the gel is as followed:

<table>
<thead>
<tr>
<th>% Acrylamide</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide/Bis</td>
<td>5.25 ml</td>
<td>7 ml</td>
<td>8.75 ml</td>
<td>10.5 ml</td>
</tr>
<tr>
<td>Lower Stock</td>
<td>7 ml</td>
<td>7 ml</td>
<td>7 ml</td>
<td>7 ml</td>
</tr>
<tr>
<td>H2O</td>
<td>15.75 ml</td>
<td>13.6 ml</td>
<td>11.8 ml</td>
<td>10.1 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>200 μl</td>
<td>200 μl</td>
<td>200 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>30 μl</td>
<td>30 μl</td>
<td>30 μl</td>
<td>30 μl</td>
</tr>
</tbody>
</table>

2. Load 10-20 μl of samples to the gel, and run at 120 V for 2 hours.

3. Semi-dye transfer at 75 mA per gel for 45 minutes.

Western Blot

1. Block the membranes with 10% (w/v) nonfat dry milk in TRIS-buffered saline containing 0.1% Tween20 (TBST) for 30 minutes.
2. After blocking, incubate the membranes with primary antibodies diluted in blocking solution for desirable time.

3. Wash with TBST for 5 minutes, and repeat for 3 times.

4. Incubate with secondary antibodies diluted in blocking solution for 1 hour.

5. Wash with TBST for 5 minutes, and repeat for 3 times.

6. Detect protein with ECL kit. Mix solution A and B at 1:1 ratio, and add to the membranes. After incubate for 1 minutes, the membrane visualized through exposed to film.
APPENDIX C
IMMUNOCYTOCHEMISTRY PROTOCOL

1. Cells are fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature.

2. Non-specific antigen sites are blocked with PBS containing 10% horse serum and 0.1% Triton X-100.

3. Cells are incubated with primary antibodies in PBS containing 1% horse serum for desirable time.

4. Rinse the cells with PBS for 5 minutes, and repeat the wash three times.

5. The cultures are incubated with goat AlexaFluor 488 (1:250 in PBS) or goat AlexaFluor 527 (1:150 in PBS) for 1 hour.

6. Cultures are counterstained with Hoechst 33342 (1:10000) for the visualization of nuclei and phalloidin (1:40) for actin filament.

7. Immunofluorescence was detected with Nikon TE 2000 inverted phase microscope, and analysis with NIS-Element software.
APPENDIX D
TIME-LAPSE PHOTOGRAPHY PROTOCOL

1. Bovine satellite cells are cultured at ECL-coated glass bottom cell culture dishes in growth medium for 24 hours until cells are attached to the surface.

2. The culture dishes are labeled and transferred to incubation chamber (37 °C and 5% CO2) linked to LiveCell™ System (Pathology Devices Inc, MD).

3. The time-lapse images were captured at 20X phase-contrast objective, with NIS-Element software (command: Acquire> capture timelapse> capture automatically> interval=5 min, duration=16 h).

4. The culture dishes are fixed and analyzed with immunocytochemistry.

5. The time-lapse movies are analyzed with NIH Image J software (command: Plugins> stacks> manual tracking). Distance of travel, velocity of each cell, and travel path are analyzed.
APPENDIX E
TRANSWELL MIGRATION ASSAY PROTOCOL

1. Transwell inserts (24-well, pore size 8 μm, Corning Inc., MA) are coated with ECL matrix for 1 hour, and then seeded with 3000 cells in 200 μl heat-inactive growth medium.

2. Heat-inactive growth medium with or without treatment are added to the lower chamber and served as a chemotactic agent.

3. After 4 or 24 hours of incubation, non-migrating cells are wiped from the upper side of the membrane, and cells on the lower side are fixed with 4% paraformaldehyde for 15 minutes.

4. Migrating cells are stained with Hoechst 33342 to calculate migrating percentage.
LIST OF REFERENCES


Edmondson, D. G. and E. N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. 3:628-640.


Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72.


BIOGRAPHICAL SKETCH

Ju Li was born in Harbin, People’s Republic of China. She graduates from Nankai University (Tianjin, P.R.China) with her B.S. in life science in September 2003. Following graduation, Ju Li worked as a research assistant for Dr. Chen in Centers for Disease Control of China. In January 2007, Ju Li got her MS degree under Dr. Sally E. Johnson from the University of Florida. Following that, Ju Li continues her Ph.D. program in Dr. Sally E. Johnson’s laboratory in UF. Ju Li currently resides in Gainesville, Florida, with her husband, Changhao Bi.